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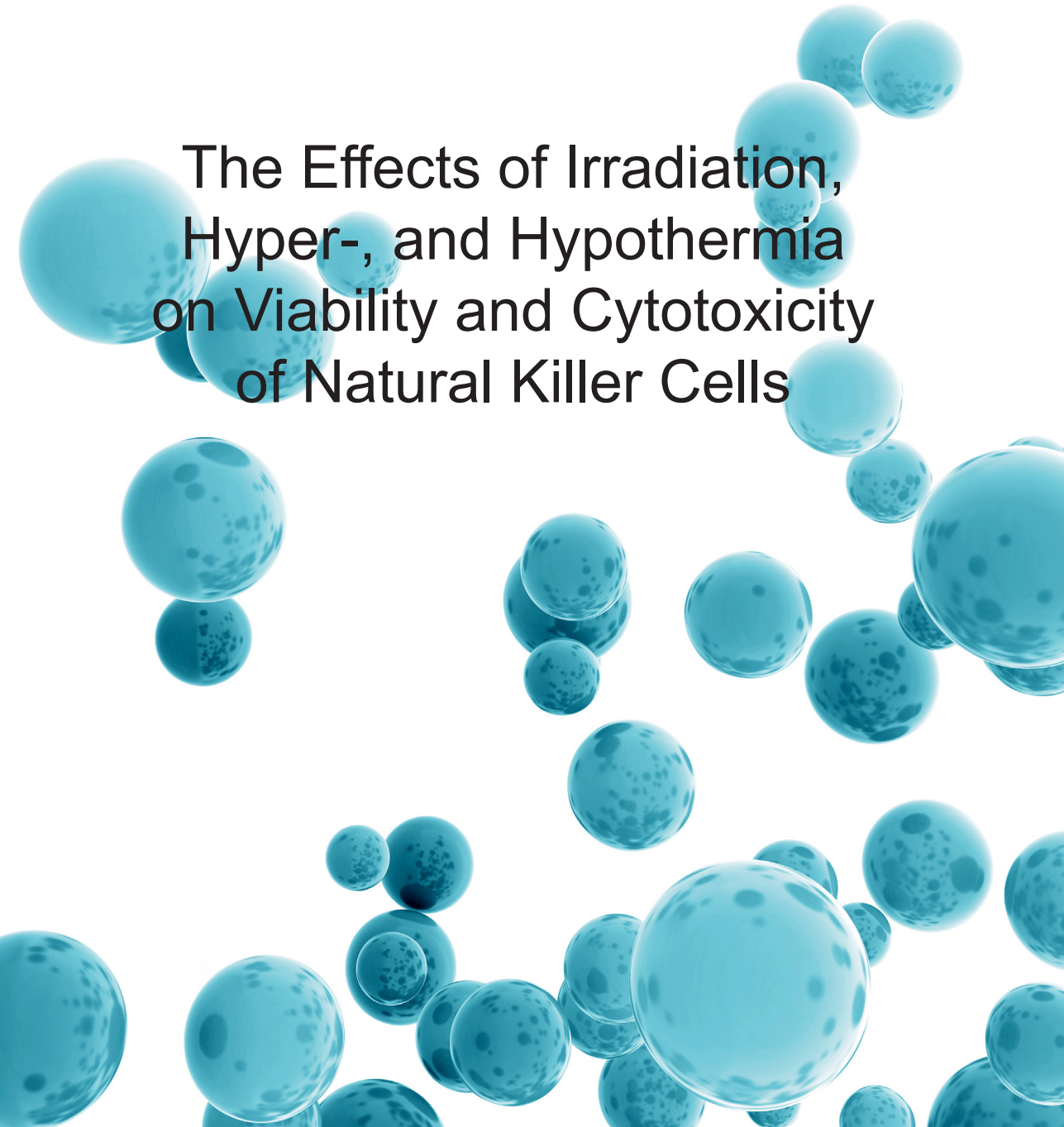
TENHO HIETANEN

The Effects of Irradiation, Hyper-, and Hypothermia on Viability and Cytotoxicity of Natural Killer Cells



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ACADEMIC DISSERTATION
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Dedicated to my considerably tolerant family

Da steh' ich nun, ich armer Tor,

Und bin so klug als wie zuvor!

(Johann Wolfgang von Goethe,
Faust, Faust Monolog)

Errata list

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Title of thesis:

The Effects of Irradiation, Hyper-, and Hypothermia on Viability and Cytotoxicity of Natural Killer Cells

Thesis page	Line	Original text	Corrected text
19	26	ifferentation	differentation
27	4	Barnet	Burnet
28	10	Studie	Studies
29	21	incident, a photon	incident photon
38	29	$\overline{D} = -\int_0^\infty D \times \div \frac{d(\boldsymbol{S}(D))}{dD} \times dD$	$\overline{D} = -\int_0^\infty D \times \frac{d(\boldsymbol{S}(D))}{dD} \times dD$
50	17	MCH	MHC
62	32	by expressing	by expressing $CD16^+$
71	3	MDCCs	MDSCs

Abstract

Natural killer (NK) cells are an important piece of the first line of defense against cells with altered or absent self-structures like major histocompatibility complex (MHC) class I molecules. Such cells include neoplastic cells, microbial infected, chemically or physically altered, and injured cells. The functions of NK cells are regulated by several factors, including interferon (IFN) α, β, γ, and interleukin-2 (IL-2).

The most-used cancer therapies include surgery, radiation, chemotherapy, and hormonal, targeted, and immunological therapies. In addition, hyperthermia and hypothermia are applied in some cancer types. All the treatment modalities affect in various ways the activation or inhibition of the natural killer (NK) cells' viability and cytotoxicity against tumor cells. In this series of investigations, the effects of radiation, immunological, and thermal treatments as well as their combinations on NK cells were studied in vitro. In addition, one purpose of this study was to find out how to minimize, prevent, or restore damages induced by irradiation and hyperthermia to NK cells.

Several NK cell isolation methods were tested to enrich non-selected and highly purified $CD56^+$ and $CD16^+$ NK cells in order to get sufficient yield, purity, viability, and cytotoxicity. The viability and cytotoxicity of NK cells were measured using various methods. The enriched NK cells were irradiated with single and fractionated irradiation using a very wide range of doses. Some standard radiobiological parameters were calculated to describe the effects of irradiation on the viability and cytotoxicity of NK cell populations. The ability of recombinant IFN α, β, γ, and IL-2 to prevent and restore radiation-injured NK cell viability and cytotoxicity was examined. Furthermore, the effects of IFNs and IL-2 on radiobiological characteristics of irradiated NK cells were examined.

NK cells were incubated at different temperatures and for different lengths of time. Recombinant IFN α, β, γ, and interleukin-2 were used to prevent and restore thermal damages. In addition, the effect of the combination of irradiation and hyperthermia on cytotoxicity was examined. The capability of IL-2 in preventing and restoring the NK cell–killing capacity in this combination was studied.

The studies showed that the NK cell–enrichment methods used influenced the yield, purity, viability and cytotoxicity, and radiation sensitivity of the enriched NK cells. The purity of $CD56^+$ and $CD16^+$ NK cells was significantly higher than that of non-selected NK cells. However, the viability and cytotoxicity of $CD56^+$ and $CD16^+$ NK cells were significantly lower when compared with non-selected NK cells. No recovery from irradiation damages was observed. There was a clear inter-individual variation in the yield, cytotoxicity, and radiation sensitivity among the NK cells from different human donors. Small to medium doses of irradiation enhanced NK cell cytotoxicity.

According to the radiobiological linear-quadratic model, the cytotoxicity and viability parameters of NK cells were similar to the acutely responding tissues. The mathematical models used reflected the different parts of the radiation dose-effect curves and the radioprotective effects of cytokines used. IFNs and IL-2 influenced the parameters of irradiated NK cells.

IFN α and β showed no dose-dependent effect on viability and cytotoxicity when used before or after irradiation. IFNγ had a significant radioprotective effect when used in very high concentrations before irradiation. In contrast to IFNs, IL-2 had a significant dose-dependent effect on NK cell cytotoxicity given either before or after irradiation. No combinations of IL-2 and IFNs showed better protection than did IL-2 alone. Splitting the radiation dose in various fractions and intervals protected the cytotoxicity of NK cells primarily at the 30 Gy level.

In thermal treatment studies, hypothermia between 31°C and 37°C showed no significant change in the cytotoxicity, which started to decrease significantly between 41°C and 42°C depending on the heating time. Heating NK cells for 180 min at 42°C abolished the cytotoxicity almost completely. The cytotoxicity recovered mainly during the first 24 h, depending on heating temperature and time. IFNs had no dose-dependent effect on the recovery, whereas IL-2 was able to restore the cytotoxicity completely, depending on the IL-2 concentration, heating temperature, and time. IL-2 was equally effective in increasing NK cell cytotoxicity when used before, during, or after the thermal treatment.

When combining irradiation and heating, there was no significant difference in the cytotoxicity when the heating or irradiation was given first. IL-2 was able to recover the cytotoxicity after this combination of heating at 42°C and irradiation, depending on heating time. The effect was significantly better when IL-2 was given before the combination of radiation and thermal treatment. Thus, IL-2 is able to prevent and recover the cytotoxicity of NK cells from damages induced by irradiation and hyperthermia as well as their combinations.

According to these in vitro studies, the selection of NK cell enrichment methods is important for reliable in vitro results. In accordance with this and other studies, the daily irradiation doses of 2 Gy activate NK cell cytotoxicity in vitro and in vivo. In vivo fever-range hyperthermia also enhances the cytotoxicity. IL-2 was a potent NK cell activator in vitro. Based on these results, the combination of radiation, hyperthermia, and IL-2 therapy might be a theoretical base for planning future clinical studies. However, the translation of in vitro findings to in vivo situations should be done cautiously. In vivo, a vast amount of activating and inhibiting factors influence NK cell viability and cytotoxicity against tumor cells.

Tiivistelmä

Luonnolliset tappajasolut (NK) ovat tärkeänä osana ensilinjan puolustuksessa soluja vastaan, joilla on muuntuneita taikka puuttuvia kudosten suuria yhteensopivuuskompleksi (major histocompatibility complex (MHC)) luokka I molekyylejä. Sellaisia soluja ovat mm. syöpäsolut, mikrobien infektoimat, kemiallisesti taikka fyysisesti muuttuneet tai vahingoittuneet solut. NK solujen toimintaa säätelevät useat tekijät, mm. interferonit (IFN) α, β, γ ja interleukiini -2 (IL-2).

Syövän hoitoon käytetään yleisimmin leikkauksia, sädehoitoa, kemoterapiaa, hormonaalisia, uusia biologisia ja immunologisia hoitoja. Hypertermiaa ja hypotermia käytetään joissakin tapauksissa. Kaikki nämä hoitotavat vaikuttavat eri tavoilla luonnollisten tappajasolujen elossaolokykyyn ja kykyyn tappaa syöpäsoluja, joko estävästi taikka edistävästi. Tässä tutkimussarjassa on selvitetty sädetyksen, immunologisten ja lämpökäsittelyjen sekä niiden yhdistelmien vaikutusta luonnollisiin tappajasoluihin in vitro. Näiden tutkimusten keskeisiä tarkoituksia oli selvittää, kuinka minimoida, estää taikka korjata säde- ja hypertermiahoitojen aiheuttamia vaurioita NK soluille.

Useita NK solujen rikastusmenetelmiä testattiin, jotta löydettäisiin menetelmät, joilla saadaan sekä valikoimattomia että erittäin puhtaita $CD56^+$ja $CD16^+$ tappajasolujen alalajeja tarpeellisiä määriä, puhtaina, elossaolevina ja kykenevinä tappamaan syöpäsoluja. NK solujen elossaolo- ja tappokyky mittattiin useilla menetelmillä. Rikastettuja NK soluja sädetettiin yksittäisillä taikka fraktioiduilla sädeannoksilla käyttäen laajaa annosaluetta.

Sädetyksen vaikutusta eri NK soluryhmien elossaolo- ja tappokykyyn kuvattiin yleisesti käytössä olevilla sädebiologisilla tunnusluvuilla. IFN α, β, γ ja interleukiini-2:n kykyä estää tai korjata NK solujen elossaolo- ja solujen tappokyvyn sädetyksestä saamia vaurioita tutkittiin. IFN α, β, γ ja interleukiini-2:n vaikutuksia sädetettyjen NK solujen sädebiologisiin tunnuslukuihin selvitettiin

NK soluja käsiteltiin eri lämpötiloissa eripituisia aikoja. IFN α, β, γ ja interleukiini-2:n kykyä estää taikka korjata lämpökäsittelyn aiheuttamia vaurioita tutkittiin. Lisäksi sädetyksen ja lämpökäsittelyn yhteisvaikutusta NK solujen

tappokykyyn selvitettiin, samoin kuin IL-2:n kykyä estää taikka korjata näitä vaurioita.

Tutkimukset osoittivat, että NK solujen rikastusmenetelmät vaikuttivat saatujen solujen määrään, puhtauteen, elossaoloon ja tappokykyyn sekä sädeherkkyyteen. Korkea-asteisesti puhdistettujen $CD56^+$ ja $CD16^+$ NK solualalajien puhtausaste oli tilastollisesti merkitsevästi korkeampi kuin valikoimattomien NK solujen. Kuitenkin $CD56^+$ ja $CD16^+$ alalajien elossaoloaste ja sytotoksisuus olivat merkitsevästi alhaisempia kuin valikoimattomien NK solujen. Toipumista sädetyksestä ei havaittu. Eri verenluovuttajien välillä oli selviä henkilökohtaisia eroja rikastettujen solujen määrässä, tappokyvyssä ja sädeherkkyydessä. Pienet - keskisuuret sädeannokset aktivoivat NK solujen sytotoksisuutta.

Radiobiologisen lineaaris-neliöllisen (LQ) mallin mukaan NK solujen sytotoksisuus ja elossaololyky vastasivat akuutisti sädetykseen reagoivia kudoksia. Eri matemaattiset mallit heijastivat parhaiten eri osia sädetyksen annos-vaste käyrissä ja käytettyjen sytokiinien säteilyltä suojaavaa tehoa. IFN α, β, γ ja interleukiini-2 vaikuttivat sädetettyjen NK solujen radiobiologisiin parametreihin.

IFN α´lla ja β´llä ei havaittu annosriippuvaista tehoa solujen elossaolokykyyn ja sytotoksisuuteen käytettäessä niitä ennen tai jälkeen sädetyksen. IFNγ´lla oli merkitsevä säteilyltä suojaava teho, jos sitä käytettiin erittäin suurina pitoisuuksina ennen sädetystä. IL-2 sitävastoin suojasi merkitsevästi annoksesta riippuen sytotoksisuutta, käytettiinpä sitä ennen taikka jälkeen sädetyksen. Mikään interferonien ja IL-2:n yhdistelmä ei ollut tehokkaampi kuin IL-2 yksinään. Kokonaissädeannoksen jakaminen erilaisiin osiin, fraktioihin, ja annettuna eripituisin tauoin suojasi NK solujen sytotoksisuutta, erityisesti 30 Gy kokonaisannoksissa.

Lämpöhoitotutkimuksissa hypotermia välillä 31°C-37°C ei vaikuttanut merkitsevästi sytotoksisuuteen. Se laski merkitsevästi vasta välillä 41°C ja 42°C. 42°C:ssa sytotoksisuus katosi melkein täysin lämmitysajasta riippuvalla tavalla. Sytotoksisuus palasi etupäässä 24 tunnin kuluessa lämpötilasta ja lämmitysajasta riippuvalla tavalla. IFN:t eivät vaikuttaneet toipumiseen annoksesta riippuvalla tavalla, kun taas IL-2 palautti sytotoksisuuden täydellisesti riippuen IL-2 pitoisuudesta, läpötilasta ja lämmitysajasta. IL-2 oli yhtä tehokas, annettiin sitä ennen lämmitystä, sen aikana tai sen jälkeen.

Jatkossa NK solut altistettiin sädetyksen ja hypertermian yhdistelmälle. Näiden käsittelyjen antojärjestys ei vaikuttanut sytotoksisuuteen. IL-2 kykeni palauttamaan sytotoksisuuden näiden yhdistelmien aiheuttamista vaurioista 42 °C asteessa riippuen lämmitysajasta. Sytotoksisuuden palautuminen oli merkitsevästi parempi,

jos IL-2 annettiin ennen sädetyksen ja hypertermian yhdistelmää. IL-2 kykeni estämään ja korjaamaan sädetyksen ja hypertermian sekä niiden yhdistelmien NK solujen sytoksisuudelle aiheuttamia vahinkoja.

Näiden tutkimuksien mukaan NK solujen rikastamismenetelmillä on merkitys luotettavien in vitro tutkimustulosten kannalta. Tämän ja muiden tutkimuksien mukaan päivittäiset 2 Gy sädeannokset lisäävät NK solujen sytotoksisuutta sekä in vitro että in vivo. IL-2 aktivoi NK soluja voimakkaasti in vitro. Teoreettiselta kannalta katsoen sädetyksen, hypertermian ja IL-2 yhdistelmä saattaisi olla pohjana kliinisille syöpätutkimuksille tulevaisuudessa. Kuitenkin in vitro havaintojen soveltaminen in vivo tilanteisiin tulee tehdä varovaisuudella sillä in vivo suuri määrä aktivoivia ja estäviä tekijöitä vaikuttavat NK solujen elossoloon ja kykyyn tuhota syöpäsoluja.

Contents

List of original publications

This doctoral dissertation is based on the following original publications, which are later referred to in the text by their Roman numerals (I–IV):

I Hietanen T, Pitkanen M, Kapanen M, Kellokumpu-Lehtinen PL. Post-Irradiation Viability and Cytotoxicity of Natural Killer Cells Isolated from Human Peripheral Blood Using Different Methods. Int J Rad Biol, 2016; 92(2):71-79.

II Hietanen T, Pitkanen M, Kapanen M, Kellokumpu-Lehtinen PL. Effects of Single and Fractionated Irradiation on Natural Killer Cell Populations: Radiobiological Characteristics of Viability and Cytotoxicity in Vitro. Anticancer Res, 2015; 35(10):5193-5200.

III Hietanen T, Kellokumpu-Lehtinen PL, and Pitkanen M. Action of Recombinant Interferons and Interleukin 2 in Modulating Radiation Effects on Viability and Cytotoxicity of Large Granular Lymphocytes. Int J Radiat Biol., 1995; 67(2): 119-126.

IV Hietanen T, Kapanen M, and Kellokumpu-Lehtinen, PL. Restoring Natural Killer Cell Cytotoxicity After Hyperthermia Alone or Combined with Radiotherapy. Anticancer Res, 2016; 36(2):555-563.

Abbreviations

Ab	Antibody
ADCC	The antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AIF	Apoptosis-initiating factor
AKT	Protein kinase B (PKB), also known as AKT, a serine/threonine-specific protein kinase
αGalCer	α-galactosylceramide
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
AP	Alternative pathway
AP-1	Activator protein-1
APC	Antigen-presenting cell
ATM	Ataxia teleangiectasia
ATP, ADP, AMP	Adenosine tri-, di-, mono phosphate
AUC	Area under the curve
B cell	B lymphocyte (bone marrow–derived lymphocyte)
BAFF	B-cell activating factor of the TNF family
Bcl-2 gene family	Gene family of apoptosis regulating proteins
BCR	B cell receptor
Bid	BH3 interacting-domain death agonist
BM	Bone marrow
C	Complement
CAR	Chimeric antigen receptor
CC121	Clonal complex CC121
CCR7	CC chemokine receptor type 7
CD	The cluster of differentiation (cluster of designation or classification determinant)
CDC	The complement-mediated cytotoxicity

CDKN1A	Cyclin-dependent kinase N1A
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COX2	Cyclooxygenase 2
CP	Classical pathway
CR	Complete response
CT	Computed tomography
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXC, CC, C, CX_3	Guanine nucleotide-binding protein
CXCL10	C-X-C motif chemokine 10
CXCL9	Chemokine (C-X-C motif) ligand 9
CRS	Cytoreductive surgery
$\overline{D}$	Mean inactivation dose
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DISC	Death initiating signaling complex
DNA	Deoxyribonucleic acid
dNK	Decidual NK cell
EDTA	Ethylenediaminetetraacetic acid
EORTC	The European Organization for Research and Treatment of Cancer
EP3	Prostaglandin E_2 receptor 3 (EP_3)
ETP	Early intrathymic precursor
Fas	Apoptosis antigen 1(APO-1 or APT), cluster of ifferentiation 95 (CD95), or tumor necrosis factor receptor superfamily member 6 (TNFRSF6)
Fcγ	Crystallizable fragment gamma
FITC	Fluorescein isothiocyanate
FL	Tyrosin kinase receptors flt3
FOXP3	Forkhead box P3, scurfin
FSRT	Fractionated stereotactic radiotherapy
G1	Gap1 phase of the cell cycle
G2	Gap 2 phase of the cell cycle
GM-CSF	Granulocyte-macrophage colony-stimulating factor

G-CSF	Granulocyte-colony stimulating factor
GM-CSF (GM-Φ)	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft versus host disease
Gy	gray (1 Joule/kg=1m^2/s^2)
H2A	Histone H2A
H2AX	H2A histone family, member X
HER	Human epidermal growth factor receptor
HEV	High endothelial venules
HIF-1	Hypoxia-inducible factor 1
HIPEC	Hyperthermic intraperitoneal chemotherapy
HLA	Human leukocyte antigen
HPC	Hematopoietic stem cell
HR	Homologous recombination
HSCT	Allogeneic hematopoietic stem cell transplant
HSF1	Heat shock factor 1
HSP	Heat shock protein
HT	Hyperthermia
ICAM1	Intracellular adhesion molecule-1
IDC	Interstitial dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL2R	Il-2 receptor
IL-2RP gene	Human interleukin 2 receptor β-chain gene
ILC	Innate lymphoid cell
imDC	Immature dendritic cell
iNK	Immature natural killer cell
iNKT	Invariant natural killer T cell
ITAM	Immunoreceptor tyrosine-based activation motif
KIR	Killer-cell immunoglobulin-like receptor
KIR-HKA	Killer immunoglobulin-like receptor–human leukocyte antigen
KL	C-kit ligand
LAK cell	Lymphokine activated killer cell
LET	Linear energy transfer

LFA-1	Lymphocyte function–associated antigen 1
LGL	Large granular lymphocyte
LP	Lectin pathway
LPS	Lipopolysaccharide
L-Q model	Linear-quadratic model
LTR	Lattice radiation therapy
M	Mitosis phase of the cell cycle
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MBL	Mannan-binding lectin
M-CSF (M-Φ)	Macrophage colony-stimulating factor
MDSC	Myeloid derived suppressor cell
MeV	Mega electron volt
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
MIU	Million international units
MRI	Magnetic Resonance Imaging
MTp53	Mutant or absent p53
MΦ	Macrophage
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NK cell	Natural killer cell
NKC	Natural killer complex
NKG2A, B, C, D, E	The natural killer group2 member A, B, C, D, E receptor
NKp46	Natural killer (NK) cell–specific surface molecule, CD335
NKT	Natural killer T cell
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	neural nitric oxide synthase
O_2	Oxygen
p53	Tumor protein p53, protein 53, cellular tumor antigen p53

PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PDGF	Platelet derived growth factor
PD-L1, 2	Programmed cell death protein ligand 1, 2
PGE2	Prostaglandin E_2
PGF2	Prostaglandin F_2
pH	Pondus hydrogenii, power of hydrogen
PI	Propidium iodide
PI3K	Phosphor-inositide-3-kinase
PKC	Protein kinase C
PR	Partial response
pre-DC1	Monocyte precursor
pre-DC2	Plasmocytoid precursor
PRM	Pattern recognition molecule
PRR	Pattern recognition receptor
RAF	Rapidly accelerated fibrosarcoma
RAS	RAS superfamily is a protein superfamily of small GTPases
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
r-PE	R-phycoerythrin
RTK	Receptor tyrosine kinase
S	Synthesis phase of the cell cycle
SBRT	stereotactic body radiation
SD	Stable disease
sIL-6Rα	Soluble form of the IL-6 receptor, α subunit
SNS	Sympathetic nervous system
STL	Secondary lymphoid tissue
T cell	T lymphocyte (thymus-derived lymphocyte)
T	The repopulation overall time
T:E	Target-effector relation
TAM	Tumor-associated macrophages
TCC	Terminal complement complex
TCR	T cell receptor

TGF	Transforming growth factor
Th1,2	Helper T cell 1 and 2
TIL	Tumor infiltrating lymphocytes
TINK	Tumor infiltrating NK cell
Tk	Onset time of repopulation
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
Tp	Doubling time of tumor tissue
Treg	Regulatory T cell
TTM	Targeted temperature management
VCAM-1	Vascular cell adhesion protein 1
VV-PISH	Veno-venous perfusion-induced systemic hyperthermia
WTp53	Wild type of protein 53

1 Introduction

Wilhelm Conrad Röntgen discovered X-rays in 1895 (Rontgen 1896). By 1896, an advanced breast cancer was being treated using X-rays (Grubbe 1947, Bernier et al. 2004). Since then, radiotherapy has developed into a high-technology therapy and one of the most important non-surgical means of treating cancer. Today, irradiation can be administered using direct ionizing (electrons, protons, α and π particles) or indirect ionizing radiation (X- and γ- radiation) as well as neutrons; lately, it has also been administered using heavy particles or radioactive substances. It can be applied in external (teletherapy) or internal form (brachytherapy) (Bernier et al. 2004).

When cells are irradiated, the absorbed energy deposits in the cells result in water radiolysis and damages to intracellular molecules, the most critical being DNA single- or double-strand breaks. Also, various proteins and subcellular organelles including mitochondria and signal transduction pathways are affected.

The irradiation damage may be instantly lethal (Schmidt-Ullrich 2003). In the case that the cell is sublethally injured, it begins reparative processes via specific enzymes and other cytoprotective mechanisms. The proportion of surviving cells is a function of the radiation dose used (Bedford 1991, Schmidt-Ullrich 2003). To be able to compare cell death and survival of various cell types irradiated with different doses, several mathematical models have been developed. Because the irradiation hits in a cell are statistically distributed, all of the survival models are based on stochastic calculations. In clinical dose planning systems, the so-called linear-quadratic (LQ) model is the most used (Kellerer and Rossi 1971, Kellerer and Rossi 2012, Bentzen 2009)

Even in the early days of radiotherapy, it was understood, due to the observed side effects, that irradiation affects both healthy and cancerous cells. In 1900, Thor Stenbeck in Stockholm divided the total dose needed to treat skin cancer into small daily fractions (Bernier et al. 2004). Accumulating studies showed that this so-called fractionated radiotherapy made it possible to increase the radiation dose and to

have better treatment results. Concomitantly, the rate of side effects dropped (Fletcher 1988, Bernier et al. 2004).

For many decades, it has been known that the radiation sensitivity of various cell types and of cells of the same type in different individuals may differ considerably. The same is true for cancer cell types and even within the same cell type in an individual patient. Variations of radiosensitivity are connected with tumor suppressor gene p53 and its mutations (Fertil et al. 1984, Williams et al. 2008). Also, p53-independent mechanisms have been discovered (Bristow et al. 1996).

Resulting from the development of radiobiological knowledge and technical radiation devices as well as dose-planning systems, about 60% of all cancer patients receive radiation therapy (Delaney et al. 2005, Prasanna et al. 2014). It has been combined with surgery, different drugs (Bernier et al. 2004), hyperthermia (Datta et al. 2016), and immunological treatments (Kalbasi et al. 2013).As a result, the therapeutic effect of combined treatments has increased when compared with single-treatment modalities, and side effects of the individual therapies have decreased (Elkind et al. 1965).

Hyperthermia, the elevation of human body temperatures, has been used in cancer therapy for at least 7,000 years. (Overgaard J. 1985). The modern era of hyperthermia began in the 1950s. Today there are several definitions of hyperthermia. According to the Kadota Fund International Forum 2004, it is a modest elevation of temperature in the range of 39-45°C. Higher temperatures are considered thermal ablation (van der Zee et al. 2000, Datta et al. 2016). Fever-range temperatures are defined as ≥ 38.3°C and high fever as ≥39.5°C (Laupland 2009).

It is known that hyperthermia can kill cells directly or indirectly by unfolding and aggregating proteins. The proteins in the cell nucleus that manage deoxyribonucleic acid (DNA) functions are critical. A heat-induced programmed cell death, apoptosis, may occur (Dayanc et al. 2008, Roti Roti 2008). Advanced hyperthermia treatment planning, thermometry, and execution have played a substantial role in increasing the use of hyperthermia and achieving better treatment results (Datta et al. 2016). It can be applied as a systemic therapy to the entire body or a specific region as well as being used as a local and as an ablative therapy. There are numerous ways to induce hyperthermia in cancer patients. Hyperthermia enhances the effects of radiation and cytotoxic therapy and the immunological responses to cancer cells including NK cells (Datta et al. 2016).

In the medical history, there are several reports of malignant tumors that disappeared without any therapy. Today it is known that those tumors were destroyed by immunological mechanisms. The concept of the immune surveillance of malignancies was introduced by Barnet in the early 1950s (Burnet 1970). Since then, considerable development in the tumor immunology has led to the so-called immuno-oncology (Hoos and Britten 2012).

The body has two principal ways of reacting to tumor cells. The phylogenetically older one is the s. c. innate immune response. The effector cell system, including natural killer (NK) and dendritic is able to react immediately, without any previous contact, against cells whose self-structures, defined by major histocompatibility complex class I (MHC class I), are altered or absent, like de novo arising or existing tumor cells. The altered self-structures of tumor cells are called tumor antigens. The response to tumor cells is a quick though unspecific one. An immunological memory will usually not develop (Melvold and Sticca 2007).

The second immunological way to resist tumor cells is the s. c. adaptive immune response. Antigenic structures of tumor cells (MHC classes I and II) are recognized by a very complex system including mainly dendritic cells and macrophages. These so-called antibody-presenting cells (APC) activate various T cell subclasses and they, in turn, activate killer T cells and B lymphocytes. The B lymphocytes produce specific antibodies to tumor cells to be attached by killer T cells and generate the immunological memory. The immune response to tumor cells may take several days; however, the response is specific. Later on, the immune response to the same tumor antigen is a quick one. The innate and adaptive systems are able to communicate with each other (Melvold and Sticca 2007).

The cell-signalling proteins regulating both innate and adaptive immune systems are called cytokines. They have both effector cell activating and inhibiting effects. In addition, they affect numerous cellular and organ functions in the body. Today, over 130 of these small glycoproteins have been identified (Vacchelli et al. 2013). According to Kyoto Encyclopaedia of Genes and Genomes, the cytokines are catalogued into nine main groups (Kanehisa et al. 2012). Among them, interleukin-2 (IL-2), a member of the large interleukin family, and interferons type I presenting numerous subclasses including IFN-α and IFN-β, type II (IFN-γ) and type III including IL-28A, IL-28B and IL-29, are important in tumor immunology and some of them are used in the therapy (Vacchelli et al. 2013).

NK cells are important in first-line defense agaist tumor cells (Smyth et al. 2001, Cheng et al. 2013). In vivo and in vitro radiation and hypothermia as well as hyperthermia influence NK cell viability and cytotoxicity. The effects are depending on irradiation and thermal dosis, either activating or inhibiting. Today, the immunological, radio-, thermo-, and thermoradiobiological features of NK cells are objects of interesting studies in vitro and in vivo (Terme et al. 2008, Cheng et al. 2013, McDowell et al. 2015)

This series of investigations are planned to elucidate the effects of single and fractionated irradiation as well as hypo- and hyperthermia on viability and cytotoxicity of NK cells and some subpopulations. Furthermore, studie were done on how to prevent or restore the damages caused by irradiation and thermal treatments using cytokines like IFNα, β, and γ, and IL-2. Finally, the capacity of IL-2 to abolish the injuries to NK cells by the combined irradiation and hyperthermia was examined. The results may provide insight on how to treat cancer patients with this combination.

2 Review of the literature

2.1 Radiotherapy and cancer treatment

2.1.1 Cellular responses to ionizing radiation

Biological effects from ionizing radiation at the cellular level can be divided roughly into four phases: physical energy transfer, radiation chemistry, biochemical processes, and cell biology, including genetics

The interactions between electromagnetic irradiation and matter are Compton scattering, pair production, proto-nuclear reactions, photoelectric effect, and Rayleight-scattering. In daily cancer radiotherapy, high energy X-rays are used. Therefore, the first two interactions above are important in daily radiation therapy (Van Lancker 1976).

In the Compton effect, the incident photon and an electron collide and a partial transfer of energy from the photon to the electron results in an ejected electron and an emission of a residual photon with reduced energy (Van Lancker 1976).

When using high photon energies >1.022 MeV, a pair production is possible. Thereby, the photon energy is converted to a mass-producing electron and positron pair or two electrons and positron. Positron is annihilated with an electron, producing two photons of 0.511 MeV (Van Lancker 1976).

In the photoelectric effect energy of the incident, a photon may be transferred to an inner electron of an atom that is ejected from its orbital. A photoelectron and an excited atom are produced (Van Lancker 1976).

The radiation may have direct and indirect effects at the cellular level. In the direct radiation effect, cellular macromolecules like DNA or proteins are directly

hit by ionizing radiation (Van Lancker 1976, Schmidt-Ullrich 2003). Besides direct radiation effects in a cell, there are numerous indirect reactions affecting essential intracellular molecules. A cell consists of 70-85% water. The water molecules undergo the most interactions with radiation, leading to the radiolysis of water (Khan 1981). The most important of the resulting ions are the radical hydrogen ion with an electron ($H\cdot$), $OH\cdot$ ion, and so called "aqueous electron" e^-_{aq}, $OH\cdot$ being most reactive. These radicals, in turn, damage cellular macromolecules. Reactive oxygen and nitrogen species (ROS and RNS) radicals may act in ionizing DNA and mitochondria. DNA damages may result in single- or double-strand breaks (Van Lancker 1976, Schmidt-Ullrich 2003)

In the case of a single strand being broken by a radiation energy deposit, the break may be fixed completely and the damage repaired. Thus, the cell may survive. This type of damage is called sublethal damage. If there are sublethal damages close to each other, they may interact, in which case the damage is called potentially lethal damage. If the rejoining of DNA strands leads to a misrepair, it results in a cell death. Breakage of a DNA double strand always causes lethal damage to the cell (Bedford 1991, Zips 2009).

Damages, depending on their degree and location, can lead to an immediate interphase cell death or necrosis, or initiate a so-called programmed cell death, also called apoptosis (Balcer-Kubiczek 2012, Maghsoudi et al. 2012). In addition, the cell can progress in the cell cycle to an arrest, leading to a senescence of the cell, continue in a delayed cell cycle, or continue dividing for some cell cycles and then die of a reproductive cell death (Bedford 1991). Over 10 different types of cell death are defined (Kroemer et al. 2009).

The ROS and RNS interact with various signal molecules essential for signal transduction cascades, affecting the stability of critical molecules. Proteasomes control degradation of short-lived proteins controlling cell-cycle progression, cell signaling, and cell death. These proteasomes functions are disturbed by radiation (Schmidt-Ullrich 2003).

Adenosine triphosphate (ATP) is the main source of energy in living cells (Okada S. 1970, Bhatt et al. 2015, Lu et al. 2015). Tumor cells use lactate to produce cellular energy by aerobic glycolysis. After irradiation using relatively low doses of about 5 Gy, mTOR (mammalian target of rapamycin), which is a regulator in cell proliferation, might be relocated to mitochondria and thus enhance oxidative phosphorylation and reduce glycolysis. As a result, mitochondrial ATP production is increased and resistance against radiation-induced cell death

enhanced (Lu et al. 2015). However, higher radiation doses reduce in a dose-dependent way the energy production of cells by affecting adenosine tri- (ATP), di- (ADP), and mono- (AMP) phosphate metabolism (Skog et al. 1983). If radiation-damaged cells are able to divide, the mutations can be inherited (Eguchi et al. 1997).

The dividing cells are more radiation-sensitive than are non-dividing ones (Suciu et al. 1975, Watanabe and Okada 1967). The cell cycle consists of five phases. In mitosis (M), a cell divides into two daughter cells. The daughter cells go on to prepare for further divisions or may be arrested and remain outside the cycling state (G_0). Then the cell synthesizes new RNA and prepares to replicate DNA (G_1). G_1 initiates DNA synthesis (S) and is followed by an interval (G_2) before a new mitosis (Zips D. 2009). The most vulnerable phase in the mitotic cell division is M phase, where chromosomes are doubled and condensed. The radiation sensitivity decreases from M to late G2, G1, early S, and late S respectively (Watanabe and Okada 1967, Skog and Tribukait 1985, Zips 2009).

There are several surveillance systems in cells capable of interrupting cell-cycle progression when the genome or spindle system is damaged. These systems are termed "checkpoints." They monitor DNA damages and regulate cell-cycle progression (Paulovich et al. 1997) Ionizing radiation slows down the cell cycle due to the activation of four DNA damage checkpoints. The checkpoints are G1 arrest, S-phase-, early G2, and late G2, respectively. It is theorized that checkpoints allow more time to repair DNA damages (Wouters and Begg 2009).

2.1.2 Radiation sensitivity

By early in the 20th century, substantial differences were detected between the radiosensitivity of normal cell types and malignant tumors (Steel G. 1989, Bernier et al. 2004, Bentzen S. 2009, Meacham and Morrison 2013). In addition, the variation of radiation sensitivity among malignancies was considerable (Fertil et al. 1984, Steel G. 1989, Meacham and Morrison 2013)

The differences between the radiosensitivity of the tumor and that of the surrounding tissues determine the therapeutic and side effects. This is the so-called therapeutic window; it defines the total irradiation dose possible to administer to a given location on a patient (Bentzen S. 2009).

Today, several genetic factors influencing this so-called intrinsic radiosensitivity are recognized. Protein 53 is a product of the p53 tumor suppressor gene. Its

normal or "wild" type (WTp53) form regulates cell division, preventing cells with damaged DNA from dividing. With ionizing radiation, it leads into a G_1 cell cycle arrest (G_1 checkpoint) or an apoptotic death. The mutant or absent form of p53 (MTp53) results in a missing or altered G_1 checkpoint and increases radioresistance (Bristow et al. 1996, Williams et al. 2008). In addition, several other factors influence the cellular radioresistance or radiosensitivity. These include mutated ataxia teleangiectasia (ATM) genes, cyclin-dependent kinase N1A (CDKN1A), 14-3-3σ (an isoform of the 14-3-3 gene), and DNA mismatch repair genes (Williams et al. 2008).

The degree of oxygenation of cells subjected to irradiation influences their radiation sensitivity (Tinganelli et al. 2013). The absorbed radiation produces highly reactive radicals (ROS and RNS) capable of damaging direct or indirect critical cellular targets including DNA. In presence of O_2, indirect lesions are fixed, whereas in the absence of oxygen the damages are chemically restored (Hewitt and Wilson 1959, Horsman M. 2009, Ma et al. 2013, Tinganelli et al. 2013).

The growing tumors induce angiogenesis for their own blood supply. Usually the generated neo-vasculature is primitive and is formed more slowly than the tumor grows. Oxygen from capillaries diffuses in tissue at approximately 100-180 μm. Beyond this distance, there is an imbalance between supply and consumption of oxygen, leading to hypoxic and even anoxic tumor regions. The hypoxic cells are more radioresistant than are well-oxygenated ones. Among several other characteristics, they are genetically more unstable and have more aggressive phenotypes (Szumiel 2015). Hypoxia evokes very many molecular pathways, most of them controlled by the hypoxia-inducible factor 1 (HIF-1) pathway (Rademakers et al. 2008).

2.1.3 Recovery from radiation

If a cell is not immediately dead, cytoprotective responses are initiated in the cell (Schmidt-Ullrich 2003). In prosurvival cellular responses, plasma membrane receptor tyrosinkinase (RTK) belonging to HER family, rapidly accelerated fibrosarcoma (RAF) receptors, and proto-oncogene RAS are involved (Schmidt-Ullrich 2003). The cells try to repair the radiation-induced damages. The lethal damages are irreparable. However, sublethal injuries are repaired within hours. The potentially lethal damages lead under ordinary circumstances to cell death. A delay

or stopping of the cell cycle after radiation increases this type of recovery by allowing more time for repair (Bedford 1991).

Two major pathways repair DNA through homologous recombination (HR) and non-homologous end joining (NHEJ) via an assembly of repair factors surrounding a DNA lesion (Huen and Chen 2010). NHEJ does not require the corresponding DNA for repair and thus is not restricted to any cell cycle phase. On the contrary, HR repair pathway requires a homologous template, for instance a sister chromatid. It might be the preferred pathway during S and G2 phases of the cell cycle (Huen and Chen 2010).

2.1.4 Apoptosis

Irradiation can induce in a cell a malignant transformation producing new mutations (Balcer-Kubiczek 2012). The cell is able to prevent this chain of events by repairing DNA damages. However, if it fails or the damage is substantial, the cell launches so-called programmed cell death, apoptosis. It can be initiated by two routes, extra- and intra-cellular (Maghsoudi et al. 2012).

In the extracellular route, tumor necrosis factor (TNF) or Fas (also known as apoptosis antigen 1 (APO-1 or APT)), cluster of differentiation 95 (CD95), or tumor necrosis factor receptor superfamily member 6 (TNFRSF6) interacts with a membrane receptor and activates death initiating signaling complex (DISC) via various caspases. They destroy cytoplasmic proteins and enter the nucleus, damaging chromatin and nucleoplasmic proteins leading to cleavage of DNA.

In the intrinsic route, caspase cleaves Bid (BH3 interacting domain death antagonist) at the outer membrane of mitochondrion. Mitochondrion leaks proteins like mitochrome C and apoptosis-initiating factor (AIF) and activates apoptosomes and caspases (Maghsoudi et al. 2012). Several genes are involved in the apoptosis, including the p53 and Bcl-2 gene family. Many lymphatic and stem cells undergo apoptosis in 3-4 hours after irradiation and prior to the first cell division (interphase cell death) (Bristow et al. 1996). In epithelial cells and tumor cells of epithelial and mesenchymal origin, apoptosis occurs later, after mitosis (postmitotic cell death) (Balcer-Kubiczek 2012).

2.1.5 Radiobiological modelling

To be able to describe and compare the radiation sensitivity of various cell types, it was necessary to develop tests and mathematical models (Lea D. 1955, Puck and Marcus 1956).

Originally, colony-forming stem cells were irradiated in vitro using various doses, and the fractions of surviving and dead cells were calculated. The cells having lethal damage were instantly dead and cells with sublethal damages were repaired. The results were presented as a cell-survival curve plotting survival fraction against radiation dose. Since then the survival curves have been described mathematically in various ways (Puck and Marcus 1956, Papworth and Hulse 1983, Joiner 2009). The hits of ionizing radiation in a cell are randomly distributed. Therefore, the low-dose region of a survival curve is stochastic in nature. The higher the radiation dose, the more the survival is predictable and thus nonstochastic or deterministic (Bolus 2001). However, most of the mathematical models describing cell survival are based on stochastic mathematic models, especially on Poisson statistics (Munro and Gilbert 1961).The first in vivo survival curve was reported by Hewitt and Wilson (Hewitt and Wilson 1959).

Single-hit single-target model: The single-hit single-target concept was developed in the 1930s and 1940s by Wyckoff and Lea (Luria and Latarjet 1947). It is presumed that cells contain radiation-sensitive regions that are necessary for their survival. The most simple assumption is that only one hit on a sensitive target causes a cell's death. This is called single-hit single-target inactivation (Lea D. 1955). Thereby it is assumed that during the irradiation there are large numbers of hits in different cells but the probability that the next hit will occur in a given cell is very small (Joiner M. 1997). Thus, for each cell, the probability of staying alive is

$$p(\text{survival}) = p(0 \text{ hits}) = \exp(-D/D_0)$$

D_0 is the dose, giving an average of one hit per target. D_0 reduces survival from one to 0.37 (i.e. to e^{-1}). D/D_0 is the average number of hits per cell.

In general, the survival curve can be described by

$$S=e^{-JD}$$

S is the surviving fraction, D is the absorbed dose (Gy), and J is hits per cell by 1 Gy. It can also be expressed by $1/D_0$. Thereby D_0 is the dose (Gy) reducing the survival fraction to 0.37 (i.e. to e^{-1}). The single-hit inactivation model describes survival curves of cells with low repair capacity and radiation damages by irradiation with high linear transfer capacity (LET) (Joiner M. 1997).

Two-hit single-target model: If it is supposed that at least two time-separated sublethal lesions in a two-track action are needed for cell inactivation, then the surviving fraction can be expressed as

$$S = e^{-JD^2}$$

(Chadwick and and Leenhouts 1973).

Single-hit multi-target model: Another survival model is the single-hit multi-target inactivation model (Luria and Latarjet 1947, Atwood and Norman 1949, Puck and Marcus 1956). It is assumed that only one hit by radiation on each of n targets in the cell induces death of the cell. According to Poisson statistics, the probability of 0 hits on a target is p (0 hits on a specific target) = $\exp(-D/D_0)$. Thus, probability of one hit to a target is p (specific target inactivated) = $1-\exp(-D/D_0)$. As there are n targets in the cell, probability that all targets are hit is

p (all n targets inactivated) = $(1-\exp(-D/D_0)\ n)$.

Thus probability of no hits is p (survival) = p (not all targets inactivated)

$$1 - (1 - e^{(-D/D_0)^n})$$

(Joiner M. 1997).

Logarithmically plotted survival curves described by the single-hit multi-target model have a shoulder at the low-dose region due to cells' capability to repair sublethal damages. Late-reacting tissues present wide shoulders and the acutely responding tissues narrow shoulders. The size of the shoulder can be described by the quasi-threshold dose (Dq), as

$Dq = D_0\ (\log_e(n))$

where D_0 is the dose (Gy) reducing the survival fraction to 0.37 (i.e. to e^{-1}) and n is number of the targets (Joiner M. 1997) (Figure 1A, p. 40).

Another factor influencing the low-dose shoulder region of radiation dose-response curve is the stimulatory response induced by low doses of an otherwise toxic agent, for instance radiation, in various organisms. This might be an evolutionary mechanism and has been shown to be connected with upregulation of the DNA repair mechanism. Mitochondrial ATP production is increased (Lu et al. 2015). The phenomenon is dependent on poly ADP-ribose and lowered thymidine kinase levels as well as production of stress proteins. Stress proteins in turn are involved in stabilizing signal-transduction, transcriptional, and/or translational machinery of the cells. In addition, prostaglandins are associated with this kind of cytoprotection (Makinodan and James 1990, Tang and Loke 2015).

Linear-quadratic model: The survival model most used today in daily radiotherapy is the s. c. linear-quadratic (LQ) model originally proposed by Lea (Lea and Catcheside 1942) and presented in a generalized formulation by Kellerer (Kellerer and Rossi 1971, Kellerer and Rossi 2012,Bentzen and Joiner 2009).

If it is assumed that a cell can be killed in two ways, with a single lethal event or by accumulation of sublethal events, then the single-hit single-target and the two-hit single-target models can be combined. This combination can be expressed as follows:

$$S=S_1S_n$$

where S_1 is the single-hit surviving fraction $e^{-\alpha D}$ and S_n is the two-hit surviving fraction $\exp(-\beta D^2)$. The combined survival fraction after a dose D is then

$$S=\exp(-(\alpha D+\beta D^2))$$

where α and β are constants.

The α component describes the cell death due to immediate irreparable lethal damages on double-stranded DNA occurring with each irradiation fraction administered and thus reflecting the total dose and not the dose per fraction, whereas the β mechanism reflects the accumulation of these damages per fraction and the dose per fraction of radiation. The α-component best fits the initial linear part of the cell-survival curve. There the cell damages increase as a function of the radiation dose. The β component describes the survival curve at higher radiation

doses. Thus, the linear-quadratic model covers the whole survival curve (Chapman 2003, Niranjan and Flickinger 2008) (Figure 1B, p. 40).

Experimental studies have shown that cells in different phases of cell cycle differ in radiosensitivity. Accordingly, the α- and β- parameters vary independently throughout the cell cycle (Gillespie et al. 1975, Chapman 2003). The single-hit mechanism is most pronounced at mitosis, whereas the double-hit mechanism is at its highest in early G1 phase. Mutant cell lines, including DNA repair-deficient cells, and lymphocytes, are killed by single-hit mechanisms. Cells of lymphoid origin die of a rapid apoptotic death. Differences between single-hit (α) and double-hit (β) radiation mechanisms in cell killing are presented in Table 1. The single-hit mechanism causes the majority of cell killing at doses higher than 2 Gy (Chapman 2003).

Table 1. Some characteristics of cell killing by the single hit (α) and double hit (β) radiation mechanisms.

	α-Mechanism	β-Mechanism	Reference
Reparability of associated lesion	none	complete	Chapman et al. (1999)
Low dose-rate	unchanged	tends to 0	Hall (2000)
Indirect effect ($OH^{\cdot}$)	about 85%	about 50%	Chapman et al. (1979)
(DMSO) for half maximum radioprotection	about 500 mM	about 150 mM	Chapman et al. (1979)
Oxygen effect (OER)	1.7 1.8	3.0 3.5	Chapman et al. (1975)
High-LET radiation	large potentiation	little change	Chapman et al. (1977)
Cell cycle radiosensitivity			Gillespie et al. (1975)
Mitosis	uniquely high	low	
Interphase	relatively constant	high in early G1 phase low in late G1 phase	
Variable radiosensitivity of tumor cells	large variation	little variation	Steel and Peacock (1989)

Adapted from Chapman (2003)

Radiation responding cells express their radiation damages within days or weeks and have a relatively high α value in comparison with β and an α/β ratio in the range 7-20 Gy or higher. Late-responding cells express the damages months or years after radiation and have a relatively low α value and an α/β ratio that ranges from 0.5 to 6 Gy (Fowler 1989).

The α/β values may vary inside a tumor due to heterogeneity of the tumor tissue, including poorly and well-differentiated cells (Meacham and Morrison 2013). The α/β of poorly differentiated cells is higher than that of well-differentiated cells. These intra-patient as well as inter-patient variations are due to the genetic mutations, and are reflected in the very high 95% confidence interval of the α/β relations (Ray et al. 2015).

The LQ model is the most used model in daily radiotherapy; however, it has been criticized. It cannot be applied to radiation doses under 1 Gy (Bentzen and Joiner 2009) and over 8 Gy, and the model may not have been sufficiently investigated for very small target volumes. The LQ model may overestimate cell death and underestimate tumor control. It does not reflect other mechanisms involved in tumor cell kill (Santacroce et al. 2013, Kondziolka et al. 2015) .

Later on, the survival models are adapted also in functional aspects of cell survival and functions (Fowler 1983).

The mean inactivation dose (area under the curve): In the 1970s it was found that in using the multi-target single-hit model the differences in the survival curves among the same histological group were much smaller in the low-dose than in the high-dose regions. In the high-dose region a small proportion of cells, varying from one experiment to another, determined the D_0 and n parameters. It was desirable to have a model with only one parameter, which was easy to calculate. Kellerer and others developed so-called mean inactivation dose $\overline{D}$ (Kellerer et al. 1976, ICRU 1979, Fertil et al. 1984, Fertil et al. 2012).

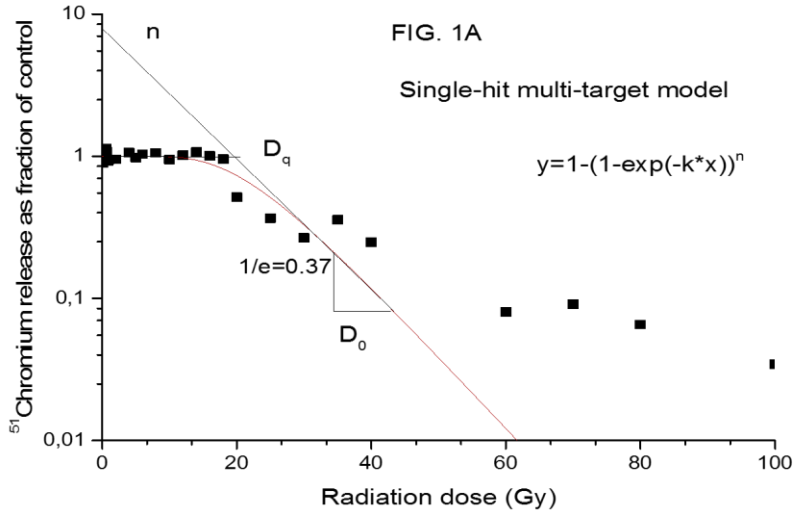
$$\overline{D} = -\int_0^\infty D \times \div \frac{d\big(\boldsymbol{S}(D)\big)}{dD} \times dD = \int_0^\infty S(D)dD$$

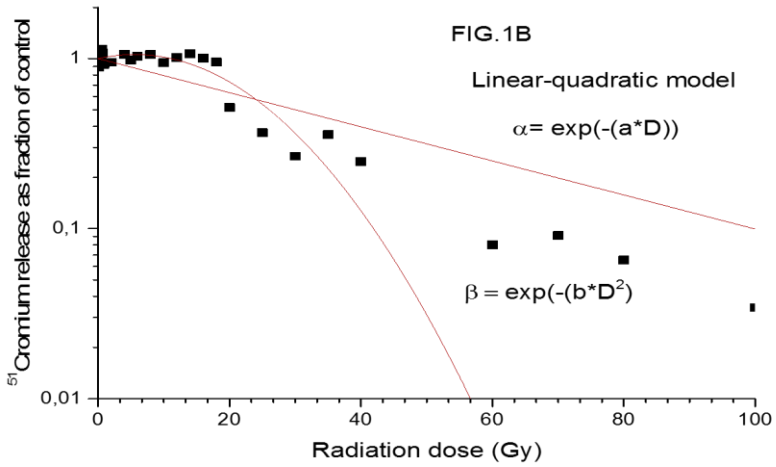
$\overline{D}$ is thus equal to the area under the survival curve plotted in linear coordinates. **S**(D) is the differential probability distribution characterized by its average dose, the so-called mean inactivation dose. S(D) is the survival probability that a dose larger than D is necessary to inactivate a cell that has

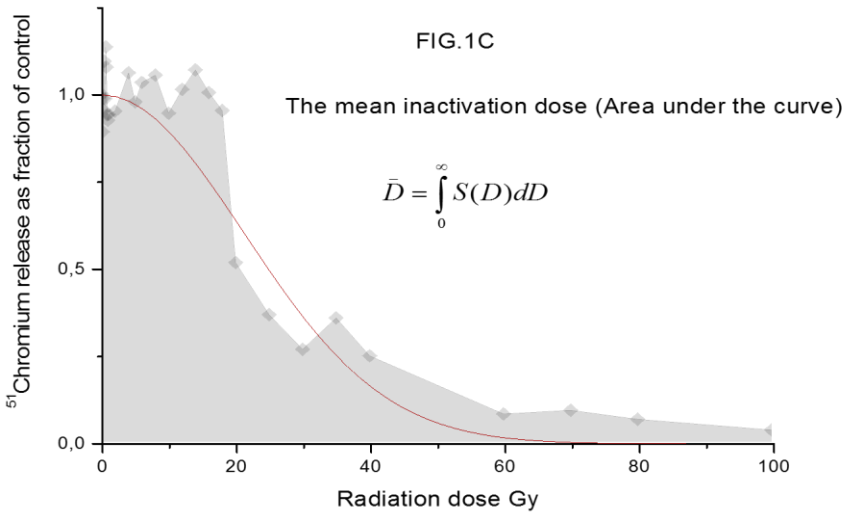
been randomly selected from the population. The mean inactivation dose proved to be better in measuring radiosensitivity than n and D_0. The variation for survival curves was much lower than for n and D_0. (Figure 1C, p. 41). ICRU recommended the mean inactivation dose to characterize the intrinsic radiosensitivity of mammalian cells (ICRU 1979, Fertil et al. 1984).

Models that are more complex are also proposed, including parameters for repair with or without misrepair components. However, the experimental validation has been difficult and the models have more of an experimental character (Papworth and Hulse 1983, Chapman 2003,).

Figure 1. Single-hit multi-target (1A), linear-quadratic (1B), and the mean inactivation dose (area under the curve, AUC) (1C) models presented in a dose-response curve of NK cell cytotoxicity test. The AUC is shown both under α/β computed curve and under actual curve of dose-response points.







Data points for presentation adopted from the material of study II of this dissertation.

2.1.6 External Radiotherapy

External radiation therapy can be given using electrons and protons or X- and γ-radiation as well as neutrons, and recently also using heavy particles (Bernier et al. 2004). A good therapeutic result in radiotherapy includes both local control and minimal late normal tissue complications. The late-responding normal tissue toxicity limits doses used in the radiotherapy (Ray et al. 2015). To increase the therapeutic results and diminish the complications, the total radiation dose can be fractionated in several ways.

2.1.7 Fractionated irradiation in daily radiotherapy

At the beginning of the radiotherapy era, radiation was given in a single dose. In the 1930s it was accepted that splitting the total dose necessary to control the tumor tissue into fractions resulted in better treatment effects and in fewer side

effects from the tumor in the surrounding healthy tissues (Fletcher 1988, BernierJ et al. 2004,). Puck and Marcus showed that splitting the irradiation killed the same proportion, though not the same number of cells, with each consecutive dose (Puck and Marcus 1956).

This phenomenon can be explained by the so-called incomplete repair model (Thames 1985). If sufficient time, for example 6-24 hours, is allowed between the fractions, the sublethal damages can be completely recovered. If this full repair interval is reduced, the total damage is increased because there are unrepaired damages left before the next fraction. Thus, the damages add up from one fraction to the next. The incomplete repair is characterized by the repair half-time (T1/2). This is the time necessary for half the maximum possible repair to take place between fractions (Joiner and van der Kogel 1997, Joiner and Bentzen 2009).

The cell-survival fraction resulting in fractionated radiation can be expressed using the LQ model, assuming a complete recovery between fractions (Joiner and Bentzen 2009). If a survival curve can be expressed using the LQ model, it follows that the incomplete repair is associated with the α but not the β component (Fowler. 1989). When cells are irradiated at low-dose rates and sublethal lesions are fully repaired, the β component tends toward zero. If the irradiation is given in a few large doses, the β component grows more pronouncedly (Chapman 2003, Bentzen 2009). Intervals of at least 6 hours are optimal for repair of radiation-induced sublethal lesions (Bristow et al. 1996). In fact, the same amount of time is required for the maximal induction of WTp53 protein (Bristow et al. 1996).

The responses of normal and tumor tissue to fractionated radiotherapy are characterized by the classic four Rs of radiotherapy: repair, reassortment, repopulation, and reoxygenation (Withers 1975):

Repair: The cellular damages recover within a few hours after radiation exposure. According to a study by Benzen et al., repair halftimes for laryngeal oedema, skin teleangiectasia, and subcutaneous fibrosis were 4.9, 3.8, and 4.4 hours respectively (Bentzen et al. 1999). Repair times for malignant cells differ considerably and may be from 4 hours to 24 hours (Withers 1975).

Reassortment: After a radiation dose, the cells in the more radiation-resistant phase of the cell cycle may survive. Within a few hours, they may progress into a more radiation-sensitive phase of the cell cycle. This phenomenon is also known as redistribution (Withers 1975, Pajonk et al. 2010,).

Repopulation: During a longer radiotherapy course, the surviving cells may proliferate. Thus, the number of tumor cells to be killed increases and the total

radiation dose should be elevated. In a classical study by Withers, the accelerated tumor repopulation was observed in patients having oropharyngeal cancers after a lag period of 4±1 weeks after initiation of radiotherapy and a therapy increment of 0.6 Gy per day was necessary to compensate the repopulation (Withers 1975).

Reoxygenation: Inside of a tumor mass are hypoxic areas, where the cells are less radiation sensitive (Hewitt and Wilson 1959, Ma et al. 2013, Tinganelli et al. 2013). When the well-oxygenated cells are first killed by radiation, the hypoxic cells may become better oxygenated and thus more radiation sensitive (Ma et al. 2013).

The repair and repopulation tends to make the tumor tissue more radiation resistant, whereas the reassortment and reoxygenation tend to make it more radiation sensitive. These factors together and the intrinsic radiation sensitivity ("the fifth R") modify the fractionated radiation effect (Steel 1997). Genetic studies show that single-dose and fractionated radiation differently alter mRNAs and miRs expression. They, in turn, coordinate immune response pathways and are associated with DNA replication, among other effects (Palayoor et al. 2014). The roles and importance of cancer stem cells in the four Rs were recently discussed by Pajonk et al. (Pajonk et al. 2010).

Standard fractionation schedule: In daily radiotherapy, the standard fractionation is defined as 1.8-2.2 Gy as single-dose per day, five days a week, for three to seven weeks. This dose range was initially selected empirically. It was assumed that larger fractions cause a steeper rise in the late adverse effects than in the rate of tumor control (Fowler 1983). Now it is known that doses of 1 Gy cause growth arrest and 2 Gy doses induce single-hit cell killing (Prasanna et al. 2014).

Altered fractionation schedules: In some instances, the therapeutic gain may be enhanced by increasing the therapeutic effect and decreasing the side effects. Which treatment modality will be selected can be estimated based on clinical experience or by calculation. For this end, radiation sensitivity of the tumor and the surrounding tissues should be known. The late- and acute-dose tolerance constraints in Gy can be determined and the appropriate total dose, treatment time, and fractionation calculated. Thereby, α/β value of the tumor, acute and late complications, the repopulation overall time (T) during the treatment, the onset of repopulation (Tk), and the doubling time of tumor tissue (Tp) are taken into consideration (Fowler et al. 1990, Yarnold et al. 2005, Owen et al. 2006).

Hypofractionation schedule: Low α/β ratio (e.g. 1-4 Gy) of the tumors reflects slow cell proliferation. Thus, it may be the same or even lower than that of the surrounding late-reacting normal tissue. The radiation can be given in larger-

than-conventional doses, and providing a longer time between the fractions will allow the sublethal damages to recover. This treatment mode, called hypofractionation, is given in doses of 3-20 Gy once a day for one to three days a week for total doses lower and total times shorter than for the conventional fractionation (Prasanna et al. 2014). Tissues with low α/β values are more sensitive to fraction size than are those with higher α/β values. Hypofractionation might cause more late side effects in slowly reacting normal tissues with low α/β values than in acutely reacting tissues with high α/β values (Prasanna et al. 2014, Ray et al. 2015).

Recent discussions have focused on the validity of the LQ model with large doses or fraction sizes. It is assumed that the lethal single-hit (αD) and the sublethal double-hit (βD^2) are not independent of each other, and the sublethal lesions will be reduced by subsequent α killing. Some investigators defend the LQ model. Thus far, the discrepancy is unresolved (Santacroce et al. 2013, Nahum 2015).

Hyper- and accelerated fractionation scheme: Some tumor cells divide faster and have a higher α/β ratio than do the late responding normal tissues surrounding the tumor (Joiner and van der Kogel 1997). The therapeutic window between tumor and normal tissues is based on differences in repair capacity, cell cycle redistribution, and relative dose rate (Horiot et al. 1992, Haslett et al. 2013).

Thus, the total radiation dose can be divided into smaller daily doses of less than 1.8-2.2 Gy, and doses can be given several times daily. The smaller fractions might reduce the risk of late toxicity and at the same time make it possible to increase the total dose. It is assumed that the higher the total dose, the better the tumor control. This is so-called hyperfractionation (Horiot et al. 1992).

In accelerated fractionation, the total dose is given over a shorter period of time, and the dose might be reduced. Thus, the repopulation of tumor cells is reduced between fractions and locoregional tumor control is improved (Haslett et al. 2013). In an EORT study with a CHART scheme, patients with head and neck cancer were treated with three 1.5 Gy fractions daily with an interfractional interval of 6 hours on 12 consecutive days including the weekend. The total treatment volume received was 37.5 Gy in 25 fractions and thereafter a smaller volume, 16.5 Gy in 11 fractions. Thus, the total dose was 54 Gy in 36 fractions. CHART did improve the therapeutic ratio relative to conventional fractionation (Bentzen et al. 1999). Accelerated and hyperfractionated modes can be combined in cases where the aim is to reduce overall treatment time (Bourhis et al. 2006). However,

hyperfractionated and accelerated radiotherapy are known to cause more acute side effects in acutely responding normal tissues with high α/β ratio than in slowly reacting tissues (Horiot et al. 1997, Bernier et al. 1998, Senkus-Konefka and Jassem 2006).

2.2 Tumor immunology

2.2.1 The immune system

All living organisms are exposed to threats from within or from other organisms. Their own new or mutated proteins, molecules, cells with mutated or inappropriately expressed gene products or presenting malignant transformation may pose danger to organisms. Also, the threats may be invading microbes, parasites, and toxins. In order to protect and not harm themselves, the organisms have to be able recognize and distinguish their own structures, "self", from foreign or harmful factors, "non-self." Subsequently, the severity of the threat must be evaluated and necessary actions performed (Delves 2006a). To this end, during the evolution the organisms have developed protection systems including an immunological one. This includes two systems, the innate and the adaptive immune systems (Melvold and Sticca 2007, Chaplin 2010,). Some of the properties of the innate and the adaptive immune systems are presented in Table 2.

Table 2. Some characteristics of innate and adaptive immunity

	Innate Immunity	Adaptive Immunity
Components	Physical barriers: e.g. Skin, mucous membranes Chemical barriers: e.g. Acidity of stomach and skin Physiological barriers: Temperature, pH, chemicals Biological barriers: Commensal microbes Cells: Phagocytes Monocytes, macrophages, neutrophils, NK cells Blood proteins: Complements: Activation via alternative and lectin pathways	Cells: Antigen-presenting cells: macrophages, B and dentritic cells Cell mediated immunity: various T cells Humoral immunity: B cells and plasma cells Blood proteins: Antibodies Complements: Activation via classical pathway
Activity type	Immediately effective	Effective after several days or weeks
Line of defense	1st line defense	Develops after exposure
Inheritance	Inherited from parents	Cannot be inherited
Specificity	Non-specific	Highly specific
Repertoire	Limited	Immense
Memory	No memory *	Long-term memory
Improvement of action	No improvement after re-exposure	Improvement after each re-exposure
Time-span	Once activated, the specific immunity remains	The developed immunity may be lifelong

The data presented in Table 2 are adapted from Melvold et al. (Melvold and Sticca 2007) and Delves et al. (Delves P. 2006b).

* Certain features of memory of NK cells (Marcus and Raulet 2013, Vivier et al. 2012) and macrophages (Shanker and Marincola 2011)

2.2.2 The innate immune system

The innate system presents a very old immune system and can be divided into two parts. The first part consists of barriers, including mechanical ones, like skin and mucous membranes, chemical barriers, such as acidity of stomach and skin, various enzymes, and antimicrobial molecules, as well as biologic barriers with commensal microbes (Melvold and Sticca 2007).

The second part of the innate system consists of pre-existing cells and molecules with an ability to react immediately to various threats. The cells have receptors and binding sites called pattern recognition receptors (PRR). In addition, the noncellular PRRs present some complement components like C3b and mannan-binding lectin (MBL) (Pio et al. 2014). Host genes regulate the PRRs without chromosomal rearrangements. Their numbers are limited to about 100 different types. One of the most important PRR families is the toll-like receptor (TLR) family, with 11 members. The response to the threats is quick but unspecific, and an immunological memory in general will not develop (Melvold and Sticca 2007).

A family of innate lymphoid cells (ILCs) includes natural killer (NK) cells that are able to recognize infected cells and their own stressed cells, including cancerous cells, and directly kill them or cause their apoptosis (Vivier et al. 2012). Recent studies show that such cells can develop a certain immune memory (Vivier et al. 2012, Marcus and Raulet 2013). Other ILC subsets produce interleukins, among others interleukin 5 (IL-5), interleukin-13 (IL-13), interleukin-17(IL-17), and interleukin-22 (IL-22). They have distinct functions in both innate and adaptive immunity (Spits and Di Santo 2011).

Further, innate immune cells comprise granulocytes like basophiles and eosinophils, which are important for defense against parasites and are involved in allergic reactions. Neutrophiles can engulf and consume bacteria (Melvold and Sticca 2007). Mast cells can destroy parasites and mediate allergic reactions. Macrophages are developed from monocytes; both regulate immune responses. Macrophages can ingest and recycle bacteria, cells, and cellular debris. Also, dendritic cells (DC) develop from monocytes (Delves P. 2006a) and are able to process structures from different pathogens into fragments and antigens and present them to cells belonging to the adaptive immune system. Therefore, they are called antigen-presenting cells (APC) (Melvold and Sticca 2007). In addition, they secrete various proinflammatory cutokines (Murray et al. 2014). Langerhans cells

and interstitial dendritic cells (imDCs) are dendritic cells of skin and mucosa (Liu 2001).

One of oldest and very important pieces of innate immunity is the complement system (Ricklin et al. 2010, Pio et al. 2014). It acts as an immune surveillance system to discriminate between health host tissue, cellular debris, apoptotic cells, and foreign intruders. It also regulates immunological and inflammatory processes and malignant cells (Ricklin et al. 2010). It enhances, or complements, the ability of antibodies and phagocytic cells to clear pathogens from an organism (Ricklin et al. 2010, Pio et al. 2014). However, some complement elements can promote tumor growth within the tumor microenvironment as well as chronic inflammation (Pio et al. 2014). In addition, complement-induced self-attacks contribute to various diseases (Ricklin et al. 2010).

The complement system consists of about 50 small proteins synthesized mainly by hepatozytes but also by macrophages, monocytes, and epithelial cells (Pio et al. 2014). The inactive precursors (pro-proteins) circulate in the body. When stimulated, proteases like C3-convertase cleave specific proteins to release cytokines, initiating a cascade of events (complement activation or complement fixation cascade) (Pio et al. 2014). There are three well-established pathways to activating complement systems. The classical pathway (CP) is activated by recognition of immunoglobulin IgM and IgG bound to target antigens via pattern recognition molecule (PRM) but also without antibodies. The lectin pathway (LP) is analogous to the classical pathway. It is activated by mannose-binding lectin (MBL) and different ficolins (Pio et al. 2014). The alternative pathway (AP) is different from the classical and lectin pathways. It does not use pathogen-binding antibodies. Complement C3 is the central molecule of AP. The AP includes also a PRM-based initiation mechanism and uses properdin. The latter recognizes pathogen and damage-associated molecular patterns (PAMP and DAMP). AP is able to distinguish self from non-self via surface expression of endogenous complement regulatory proteins. The effector functions of the complement system include phagocytosis of antigens (opsonization), attraction of macrophages, monocytes, and neutrophils promoting phagocytosis (chemotaxis), membrane rupturing of foreign cells via terminal complement complex (TCC) (cell lysis), and clustering of pathogens (agglutination) (Ricklin et al. 2010, Pio et al. 2014). Several soluble and cell-bound complement regulators control complement activity (Ricklin et al. 2010).

The innate immune response to tumors: Normal nucleated human cells express on their surface major histocompatibility complex (MHC) class I molecules, denoting that these cells are "own" or "self." Therefore, they are not usually attached by the body's own immunological effector cells. It is shown that the tumor cells produce abnormal molecules on the cell surface, so-called tumor antigens. They can express mutated normal self gene products including proto-oncogenes, tumor suppressor, or random genes. Furthermore, they can be normal cell proteins in too low or too high levels or foreign proteins to trigger an immune response (Melvold and Sticca 2007).

Natural killer (NK) cells are the most important innate effector cells (Cheng et al. 2013). NK cells have germline-encoded transmembrane pattern recognition receptors (PRR) for pathogens and damaged self-components (Shanker and Marincola 2011). NK cells have killer activation and killer inhibition receptors. Target cells express surface molecules like MHC class I polypeptide-related sequence A and B (MICA, MICB) glycoproteins. NK cells are able to identify them and bind to target cells. First, killer inhibition receptors scan the level of MHC class I on the surface of the target cell. If the MHC level of the target cell is absent or too low, the NK cell proceeds to kill the cell by releasing cytoplasmic granules containing perforin. It induces membrane perforation and a quick cell death. Perforations also allow granzyme enzymes to enter the target cell and lead to single-strand DNA breaks and apoptosis of tumor cells. Target cell killing may also be induced indirectly by secretion of IFNγ by activating other immunological cells of the antibody-dependent cellular cytotoxicity (Melvold and Sticca 2007, Shanker and Marincola 2011).

In addition to NK cells, monocyte-derived macrophages and dendritic cells are able to recognize tumor antigens and to kill tumor cells (Liu 2001, Murray et al. 2014). They release intra- and extracellular lysosomal enzymes, oxygen intermediates, and nitric oxide ROS and RNS, affecting also the tumor microenvironment. They are antibody presenting cells (APC) and are involved in maturation and activation of NK cells (Melvold and Sticca 2007). Macrophages may undergo a differentiation process, gaining features of memory (Shanker and Marincola 2011).

2.2.3 The adaptive immune system

In the adaptive immune system, the immunological response is slower than in the innate system. However, the response is more specific and intense. In addition, a specific immunologic memory is developed against the threat in question. The most important cells are the T cells and the B cells (Melvold and Sticca 2007). The DNA of the cells, using a relatively small number of genes, is able to randomly generate a vast number of different receptors. Theoretically, "the immunologic repertoire" of a person could express trillions of different varieties; in practice, it could express about 10 million. Each of the varieties can be propagated in the cell for generations to come and maintain the same specificity (Melvold and Sticca 2007).

Most adaptive immune responses are initiated in lymph nodes by APC including macrophages, dendritic, and B cells. They present protein/peptide antigens to T lymphocytes, mainly in two different ways. First, infectious organisms, debris of dead cells, and other materials ingested by APC are processed by phagosomes and lysosomes. Subsequently, they are fused with newly synthesized MCH class II molecules and exposed to T cells while in contact with the APCs. Second, the APCs may also synthesize proteins or peptides using ingested foreign proteins and load these peptides in the endoplasmic reticulum onto newly formed MHC class I molecules. These complexes are exposed to T cells that make contact with the APC (Melvold and Sticca 2007).

T cells can be divided into two categories. T cells expressing CD4 molecules react with peptides presented by MHC class II molecules, whereas T cells expressing CD8 molecules are activated by peptides presented by MHC class I molecules. Binding of both $CD4^+$ T and $CD8^+$ T cells on the APC initiates their differentiation and the synthesis of a soluble cytokine interleukin-2 (IL-2), as well as an IL-2 receptor (IL-2R). IL-2 from $CD4^+$ T cells often activates $CD8^+$ T cells. Once activated, both T cell types circulate in the body, looking for the same peptide-MHC combination that activated them (Melvold and Sticca 2007, Rothenberg et al. 2016). $CD4^+$ T cells may be further differentiated into T helper 1 (Th1) or T helper 2 (Th2). Th1 $CD4^+$ T cells are involved in delayed-type hypersensitivity and Th2 $CD4^+$ T cells regulate the activation and antibody secretion by B cells (Melvold and Sticca 2007). Tumor necrosis factor β (TNFβ) induces the development of regulatory $CD4^+$ T cells (Treg), which suppress the T cell activity (Curiel 2008).

$CD8^+$ T cells are so-called cytotoxic T lymphocytes (CTL) and circulate in the body looking for the peptide-MHC I complexes fitting their TCRs. They act directly against target cells using cell-to-cell contact and, like NK cells, secrete cytotoxic granules containing perforin into the tumor cells. Perforin damages target-cell membranes, letting granzyme molecules flow into cells. This induces single-strand DNA breaks and causes apoptosis of the target cell. The apoptotic cells undergo phagocytosis and are further processed in the immune system (Delves et al. 2006a). $CD8^+$ T cells, having had contact with specific antigens and $CD4^+$T helper cells, might develop into memory T cells. The $CD8^+$ memory T cells are heterogeneous in phenotype (Klebanoff et al. 2006).

B cells are produced in the bone marrow, from where they migrate to the lymph nodes. Primarily, they have only immunoglobulin M (IgM) on their surfaces. A naïve B cell is activated when it finds an antigen capable of binding to its surface. However, soluble IL-4 from activated Th2 cells and interaction of membrane molecules on two cells are necessary for full B cell activation and proliferation. When activated, B cells differentiate into either plasma cells or memory B cells. Plasma cells lose surface IgM expression, but secrete large amounts of soluble IgM and die within days or weeks. Memory B cells retain the surface expression of IgM. Their B cell receptor (BCR) binds to the same antigen as before, and in cooperation with Th2, they become reactivated, start to differentiate, and proliferate. Reactivated memory B cells might have two changes affecting their antibody expression. First, small DNA mutations may occur in the antigen-binding regions, leading to production of antibodies with increased intensity. The second change in the DNA may allow a shift from production of IgM to production of other antibody isotopes like IgG, IgA, or IgE, while still retaining the same antigenic specificity. The most common isotype is the IgG that, among other functions, mediates the antibody-dependent cellular cytotoxicity and increases the activity of phagocytic immune cells. The primary immune response in the adaptive immune system requires one or two weeks to grow to an effective level. The secondary response with increased activity and persistence develop in some days (Delves et al. 2006b, Melvold and Sticca 2007).

The adaptive immune response to tumors: The cytotoxic T cells (CTLs, $CD8^+$ T cells) are the main mechanism for immune surveillance in the adaptive immunity destroying premalignant and malignant cells. They may kill the cells directly or via apoptosis. Their development usually requires APCs presenting the appropriate tumor antigens. MHC classes I and II are necessary to stimulate the

CTL production and $CD4^+$ helper T cells to secrete cytokines. $CD8^+$ CTLs in turn secrete IFNγ to activate the cytotoxic macrophages and NK cells (Melvold and Sticca 2007).

B cells produce specific antibodies against tumor cell antigens, which lead to killing the corresponding antigens bearing tumor cells. The antibody-mediated killing includes the antibody-dependent cell-mediated (ADCC) and complement-mediated cytotoxicity (CDC) (Ricklin et al. 2010). Both B and T cells can develop so-called immunological memory. They can recognize again, even after considerable time, target cells with the same immunological pattern (Klebanoff et al. 2006).

2.2.4 Cooperation between innate and adaptive immune responses

The innate and adaptive immune systems are able to cooperate (Shanker and Marincola 2011, Grizzi et al. 2013). The immune effector cells, including NK cells, macrophages, $CD8^+$ T cells, and a subgroup of T cells, the γδT cells, express the natural killer group 2 member D (NKG2D) receptor. Cytotoxic T cells can be activated and exert a direct cytolytic effect against tumor cells even if they present low levels of tumor antigens. When activated, $CD8^+$ T cells in the vicinity of dormant NK cells are able to activate the dormant cells to become killer cells (Shanker and Marincola 2011). In some cases, complete tumor regression is possible only if activated $CD8^+$ or $CD4^+$and NK cells interact (Shanker and Marincola 2011). NK cells may affect the adaptive immune responses by interacting positively or negatively with the regulation of dendritic cells. In turn, dendritic cells may regulate NK cell functions (Raulet 2004).

Tumor-associated inflammation is present at all stages of tumor development, causing weakening of the anti-tumor immunoresponses (Shalapour and Karin 2015). It has been shown that independent cooperative crosstalk between adaptive and innate immunity can strengthen the host defense. Tumors are often associated with the suppression of adaptive immunity. Innate memory responses against tumors via NK cells and macrophages can renew the adaptive immune response (Foster and Medzhitov 2009).

2.2.5 Cytokines and chemokines

Cytokines are a very heterogeneous group of small signalling glycoproteins operating via specific transmembrane receptors expressed on the plasma membrane of target cells. Cytokines are produced by a great variety of distinct cells, including cells of the immune system, epithelial, endothelial, and stromal cells, as well as tumor cells (Steinke and Borish 2006, Vacchelli et al. 2013). They use autocrine, paracrine, and endocrine signalling pathways. They regulate almost all aspects of innate and adaptive immunity, cell proliferation, chemotaxis, differentiation, inflammation, elimination of pathogenes, and cell death (Borish and Steinke 2003, Steinke and Borish 2006, Vacchelli et al. 2013). Cytokines can induce the release of other cytokines, using self-amplificatory or self-inhibitory signalling cascades (Borish and Steinke 2003, Vacchelli et al. 2013). Traditionally, cytokines produced by lymphocytes are called lymphokines, cytokines mediating communications between leucocytes are interleukins, and cytokines stimulating chemotaxis are chemokines.,

Cytokines are divided into nine main groups: 1) chemokines with chemotactic activities and with several subgroups, 2) hematopoietic growth factors, 3) interleukin-1 family, 4) interleukin-10 family, 5) interleukin-17 family, 6) interferons (IFN), 7) platelet-derived growth factor (PDGF) family, 8) transforming growth factor (TGFβ) family, and 9) tumor necrosis factors (TNF) (Vacchelli et al. 2013).

Chemokines are small secreted proteins classified by their secondary structure and a distinguishing tetracysteine motif. Over 40 different human chemokines have been detected in four subfamilies: CXC, CC, C, and CX_3C, as well as over 20 guanine nucleotide-binding proteins (G proteins)-coupled receptors. Originally, they were found to be mediating the infiltration of leukocytes into inflammatory or injured tissues through vascular cells. They belong to a superfamily of polypeptide chemoattractants (Wang et al. 1998).

They induce directional migration and activation of leukocytes and regulate leukocyte adhesion, trafficking, homing, and angiogenesis. They are part of lymphopoesis and hematopoesis (Baggiolini et al. 1997). In addition, they contribute to embryogenesis and tumor progression (Wang et al. 1998, Borish and Steinke 2003) and play a role in many types of diseases (Bacon and Oppenheim 1998). Chemokines are produced by hematopoietic and non-hematopoietic cells including B lymphocytes, T lymphocytes, NK cells, neutrophils, monocytes,

endothelial cells, epithelial cells, fibroblasts, keratinocytes, and smooth muscle cells (Wang et al. 1998).

Hematopoietic growth factors: The hematopoietic growth factors comprise more than 25 cytokines controlling proliferation, differentiation, and preservation of equilibrium of hematopoietic stem cells and their compartments (McClanahan et al. 1993). The IL-2Rp gene encodes one subunit of the high-affinity receptor of IL-2, a multifunctional cytokine whose activities include antigen-specific proliferation of T cells, stimulation of B cells, macrophages, natural killer (NK) cells, lymphokine activated killer (LAK) cells, and even action on oligodendrocytes (McClanahan et al. 1993).

Granulocyte-colony stimulating factor (G-CSF) and Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulate bone marrow stem cells toward granulocytic and monocytic lineages. G-CSFs mobilize hematopoietic precursor cells from the bone marrow into the bloodstream (Thomas et al. 2002). IL-4 stimulates the differentiation of the naïve helper T cells into Th2 cells, which in turn produce additional IL-4 as well as IL-13 and immunosuppressive IL-10. Thus, the Th2 response may promote tumor growth (De Monte et al. 2011). IL-6 regulates inflammation and immunity. Some cancers produce IL-6, which is required for tumor survival (Barton 2005). IL-7 stimulates the differentiation of hematopoietic stem cells and proliferation of mature cells (Vacchelli et al. 2013). IL-12 is involved in the differentiation of naïve T cells and stimulates the function of T and NK cells. IL-12 promotes the secretion of TNFα and IFNγ, and thus inhibits IL-4 and stimulates a cytotoxic Th1 response (Trinchieri 2003). IL-15 and IL-21 regulate NK and T cell proliferation and activation (Parrish-Novak et al. 2000).

Interleukin-1 family: The interleukin-1 family includes IL-1α, IL-1β, and IL-18 and is mainly produced by macrophages. They are involved in local and systemic inflammation. IL-18 might suppress metastatic surveillance by NK cells (Terme et al. 2011, Vacchelli et al. 2013).

Interleukin-10 family: The interleukin-10 family consists of IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29. The biological effects of this family are extremely heterogeneous. IL-10 may induce consistent immunosuppression. IL-28A, IL-28B and IL-29 ("type III IFNs") might induce an antiviral state, IL-19. IL-20, IL-22, and IL-26 exert mitogenic and pro-survival effects (Kunz et al. 2006, Vacchelli et al. 2013).

Interleukin-17 family: The interleukin-17 family includes six different IL-17 variants (IL-17A-F). They are secreted mainly by helper T cells, have pro-inflammatory functions, and stimulate many other cytokines, chemokines, and prostaglandins. The role of IL-17 producing T helper cells (Th17) in tumor progression and immune response is controversial. On the one hand, the Tγδ cells activated by IL-1β and IL-23 are necessary for optimal immune response, perhaps via GM-GSF and IFNγ (Andrews et al. 2011). On the other hand, Th17 cells are immunosuppressive under influence of TGFβ, and IL-6 producing IL-17 and IL-10 (Ngiow et al. 2010, Ma et al. 2011, Vacchelli et al. 2013).

Interferons: Interferons (IFNs) are able to establish a robust antiviral state. In addition, they activate immune cells and stimulate antigen presentation to T lymphocytes, and thus take part in tumor cell recognition (Vacchelli et al. 2013). They are traditionally divided into three classes: 1) Type I, including IFNα, IFNβ, IFNε, IFNκ, and IFNω; 2) Type II, in humans IFNγ; 3) Type III, including IL-28A, IL-28B, and IL-29. IFN type I is produced by fibroblasts, monocytes, macrophages, B lymphocytes, and NK cells. IFN type II is produced by Th1, NK cells, and macrophages (Borish and Steinke 2003). IFNγ is the most important cytokine in cell-mediated immunity. Among other functions, it increases the expression of MHC I and II classes, and stimulates antigen presentation, cytokine production, and effector functions of monocytic cells, and killing capacity of NK and neutrophils (Borish and Steinke 2003).

Platelet-derived growth factor family: Platelet-derived growth factor (PDGF) consists of four PDGF isoforms (PDGFA-D), four isoforms of vascular endothelial growth factors, and six other proteins that are rather heterogeneous. PDGFs can be released by activated platelets, smooth muscle cells, activated macrophages, and endothelial cells (Pietras et al. 2003). They activate cellular responses via two distinct tyrosine kinase receptors. PDGFs affect mitogenic functions over a vast number of cell types, including the development of angiogenesis (Li et al. 2003, Vacchelli et al. 2013). PDGFs are often hyperactivated in cancer by point mutations in various receptors. Thereby they may release so-called "angiogenic switch," causing the transition of a tumor mass from a non-vascularized to a vascularized state (Bergers and Benjamin 2003, Vacchelli et al. 2013). In some instances, however, they may expand DCs and thus enhance antitumor effect (Fernandez et al. 1999, Vacchelli et al. 2013).

Transforming growth factor family: Transforming growth factor (TGFβ) demonstrates three distinct subtypes (TGFβ1-3) with at least eight additional

proteins. In normal cells, TGFβ1 are antiproliferative by upregulating the cell-cycle inhibitors like p21 and p15. They may also trigger cell death by activating apoptosis pathways (Schmierer and Hill 2007, Vacchelli et al. 2013). When overexpressed, TGFβ stimulates angiogenesis and is able to convert effector T lymphocytes into immunosuppressive Treg or Th17 cells, thus being tumor-stimulating (Blobe et al. 2000, Vacchelli et al. 2013).

Tumor necrosis factor family: The tumor necrosis factor family presents more than 15 proteins, all of them triggering cell death by binding to specific transmembrane receptors. TNFα can bind two transmembrane receptors, type I TNFR (TNFR1), found at the surface of virtually all cells, and type II TNFR (TNFR2). TNFR1 with various ligands can induce a variety of effects, including regulation of proliferation, differentiation, and cell death via apoptosis or regulated necrosis (Galluzzi et al. 2012, Vacchelli et al. 2013). TNFR ligations are also important in the development, homeostasis, and function of immune system, including DCs and even differentiation of osteoclasts (Delves et al. 2006). TNFα also plays an important role in many inflammatory diseases (Kong et al. 1999, Vacchelli et al. 2013).

For an innate immune system the chemokine interleukin-2, a member of the large hematopoietic growth factor family, is secreted by the dendritic and T cells. It is an important NK, T, and B cell activator and growth factor. It is used in cancer therapy. The interferons type I (IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω) and type II (IFNγ) are necessary in the innate system. IFNα, β, and γ are used in tumor immunotherapy; IFN-β is also used for multiple sclerosis. The co-operation of the cytokines and immune cells is extremely complex (Steinke and Borish 2006).

2.2.6 Prevention of self-destructive immune reactions

T cell control: Several mechanisms are needed to prevent the immune system from attacking self-structures. During the development of T cells within the thymus, a negative selection process forces self-reactive T cells into apoptosis. APCs with appropriate peptide and MHC but unable to provide the additional signals to activate naïve T cells lead the T cells in inactivation (Rothenberg et al. 2010). After activation, T cells develop a surface molecule CD152 that inactivates T cells, preventing excessive accumulation. $CD4^+$T regulatory T cells (Treg) inhibit the activity of other T cells directed against self-antigens (Pardoll 2012). Most

antigenic responses require balance of Th1 and Th2 activity . Th1 cells inhibit the activity of Th2 by IFN-γ production, whereas Th2 control Th1 cells by IL-4, IL-10, and T cell growth factor-β. Almost all B cell activations require participation and regulation by T cells. Thus, the random and uncontrolled production of potentially self-reactive antibodies is prevented (Melvold and Sticca 2007).

2.2.7 Possibilities of tumor escape from immune surveilance

Tumor escape from immune surveillance: There are two main pathways for tumors to escape the immune system: 1) immunogenic determinants may be lost at genetic level by deletion or mutation of a gene, at epigenetic level by silencing a gene, or at post-translational level, and 2) tumor-driven suppression of the immune response may occur by expression of immune-inhibiting molecules at the cell surface or secretion of immunosuppressive cytokines (Marcus and Raulet 2013). The balance between inhibitory and activating receptors or ligands on effector cells might be shifted in the direction of inhibition (Shalapour and Karin 2015).

Immunoediting: The relation between tumor immunological defense and tumor cells can be divided into three distinct phases: 1) the elimination of tumor cells, 2) equilibrium between tumor cell killing and emergence of resistant mutations, and 3) escape of tumor from immune control (Schreiber et al. 2011). The immune system can recognize and kill tumor cells. However, tumor cells constantly form mutations. In some cases, specific mutations eliminate epitopes necessary for recognition by T cells (Matsushita et al. 2012). In the NKG2D-dependent immune system, mutant tumor cells may have lost the expression of the necessary ligand and are therefore able to evade the immune system (Guerra et al. 2008). In addition, the NKp46 ligand may be lost (Elboim et al. 2010, Vayrynen et al. 2014). Other mechanisms causing tumor evasion include defects in costimulation or inhibition of T cells, increase of Treg cells, other suppressor cells, or IL-10 in tumors (Schreiber et al. 2011, Vayrynen et al. 2014).

Ligand shedding: Various tumor types release soluble NKG2D ligands, effecting NKG2D/NKG2D-ligand interactions. This can result in lower tumor-cell surface levels of NKG2D and reduced susceptibility to cytolysis by NK and T cells (Chauveau et al. 2010). Furthermore, the soluble ligands may prevent the interactions of NKG2D with membrane-bound ligands. In addition, soluble

NKG2D ligands may modulate NKG2D expression and downregulate NK cells (Ashiru et al. 2010, Fernandez-Messina et al. 2010).

Anergy of NK and T cells: A chronic stimulation by activating ligands like NKG2D may induce hyporesponsiveness or anergy of NK cells (Coudert et al. 2008). The same phenomenon is seen in MHC I deficient lymphoma. Long-lasting stimulation by MHC I cells results in the induction of a functionally anergic phenotype of NK cells. In mice, the anergy can be reversed by pro-inflammatory cytokines IL-12 and IL-18 (Epling-Burnette et al. 2007, Marcus and Raulet 2013).

Similarly, T cell anergy can be induced by an active imbalance between stimulatory and inhibitory B7 family members in the tumor microenvironment. B7 proteins are present on the surface of APC. They can be coactivating or coinhibiting to T cells. T cell exhaustion occurs when T cells are chronically activated at sites of chronic inflammation, such as cancer, autoimmunity, and chronic infection. The exhaustion might be mediated by the PD-1 pathway (Crespo et al. 2013).

Chronic or repetitive antigen stimulation induces corrupted memory $CD8^+$ cells with exhaustion of effector functions and proliferative capacity that might lead to a replicative senescence (Klebanoff et al. 2006).

2.2.8 Microenvironment

The solid tumors and the peritumoral stroma consist of a variety of cells, including stromal cells like fibroblasts, vascular endothelial cells, pericytes, mesenchymal cells, and extracellular matrix (Gajewski et al. 2013). Innate lymphoid cells like NK cells and natural killer T cells, dendritic cells, macrophages, neutrophils mast cells, and myeloid-derived suppressor cells are found in the microenvironment of tumors. In addition, adaptive immune cells like T and B cells are presented. All immune and non-immune cells exert positive or negative effects on host immune responses and tumor growth (Grivennikov et al. 2010, Gajewski et al. 2013).

In some cancers, the presence of activated tumor-infiltrating $CD8^+$ T cells within the tumor (tumor-infiltrating T cells, TILs) and in the peritumoral stroma has been associated with positive clinical outcome (Curiel et al. 2004, Gajewski et al. 2013). However, in metastatic melanoma, the T cell function is downregulated by IFNγ and Treg cells–dependent events. Also, T cell anergy leading to intrinsic dysfunction is observed (Gajewski et al. 2013). NK cells are active in solid tumors

and in hematologic malignancies. Circulating shed MICA or MICB ligands may induce NKG2D on effector T cells and thus diminish the NK and T cell cytotoxicity (Groh et al. 2002). Tγδ, NKT, and iNKT exert antitumor and immunomodulation effects in the tumor microenvironment via IFNγ. However, a subset of NKT suppresses the antitumor immunity via IL-13 and TGF-β (Terabe et al. 2000).

Macrophages and some myeloid cells contribute to tumor escape from immunologic surveillance. M2 macrophages are tumor-suppressive through IL-10 and TGF-β. M1 macrophages downregulate cytotoxicity via GM-CSF, IFNγ, and TLR-TLR agonists. In addition, immature myeloid cells are immunosuppressive in the tumor microenvironment (Gajewski et al. 2013). Further, fibroblasts in the tumor environment impede antitumor immune response. Also, collagen deposits may be a barrier to the T cell entry. Several angiogenic factors diminish T cell tumor infiltration (Gajewski et al. 2013).

Inflammatory microenvironment and immune response to the cancer cells: Rudolf Virchof was the first to connect tumors and inflammation (Balkwill and Mantovani 2001). Today, it is known that tumor-associated inflammation is a key feature of cancer (Grivennikov et al. 2010, Shalapour and Karin 2015). About 15-20% of cancers are based in preexisting inflammation, whereas nearly all other solid tumors have tumor-elicited inflammation.

Acute inflammation may promote antitumor immunity, whereas chronic inflammation contributes to the tumor-promoting microenvironment, to the tumor development, progression, metastatic dissemination, and treatment resistance (Shalapour and Karin 2015). The immune cells, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAM), and regulatory T cells (Treg), and the stromal cells communicate with each other. They use direct contact or cytokines and chemokines like TGFβ and IL-10. In addition, they upregulate ligands like the programmed death ligand-1 (PD-L1) (Yang 2015). The balance between inflammatory tumor prevention and promotion factors may determine the fate of the tumor (Grivennikov et al. 2010).

2.3 Immune cells in oncology in response to tumor

2.3.1 Some essential effector cells of the innate immune system in tumor immunosurveillance

***Natural killer cells and their development*:** Natural killer cells are a part of the innate immune system. In pregnancy, the fetus is a semi-allogeneic transplant. However, it is able to escape rejection by the maternal immune system. In this immune privilege, the decidual NK (dNK) cells are important. It is supposed that dNK cells have migrated from peripheral blood and acquired functional features via the decidual microenvironment, or that they are derived from immature NK cells/NK precursors ($CD34^+$) in the endometrium (Vacca et al. 2011). During the first trimester of pregnancy, NK cells represent 50-90% of decidual lymphoid cells (dNK). They support, via releasing cytokines and chemokines, the growth of spiral arteries, placentation, trophoblast migration, and tissue remodelling. Crosstalk between dNK and decidual myelomonocytic $dCD14^+$ cells induces regulatory T (Treg) cells. Tregs, in turn, create, together with dNK and myelomonocytic cells, a tolerant microenvironment at the interface between fetus and mother (von Rango et al. 2007, Vacca et al. 2011).

It is generally accepted that in adults, NK cells are bone marrow (BM) microenvironment derived. Today, it is assumed that BM-derived developmental intermediates are located within the secondary lymphoid tissue (STL) in the parafollicular T-cell-rich regions of lymph nodes. Their development occurs also in the tonsils, in Peyer's patches, and in the white pulp of the spleen before they are released as NK cells into the peripheral blood (Freud and Caligiuri 2006).

The genes encoding the NK cell development and functions are situated on human chromosome 12 in the so-called NK complex (NKC). The family of KIR genes is located on chromosome 19 in the leukocyte receptor cluster (Freud and Caligiuri 2006).

The extra uterine development of $CD34^+$ hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) to mature NK cells goes through several stages. In stage I, $CD34^+$ HPCs become $CD34^+CD122^+$ pre-NK cells via IL-2 and IL-15 induction (Colucci et al. 2003). Stage II pre-NK cells ($CD34^+CD45RA^+CD117^+CD94$) respond to IL-2 and IL-15 and differentiate further to stage III immature NK cells (iNK). They express NK-associated

antigens like CD2, CD7, CD56, CD161, 2B4, and NKp44. They are not able to produce IFNγ and mediate perforin-dependent cellular cytotoxicity against MHC I-negative cells. In addition, they lack surface receptors including CD94-NKG2 group receptors. Stage IV NK cells have acquired CD94-NKG2A expression. Thus, they can express MHC-I dependent inhibition. In addition, they express NKG2D and NKp46 receptors. They have intracellular perforin and they are able to perform perforin mediated cell killing. Further, they produce IFNγ. During the maturation of NK cells, the expression of receptor CD34 gradually diminishes and CD56 increases (Freud and Caligiuri 2006).

Numerous cytokines control the development and homeostasis of NK cells; the most important is IL-15. The others include IL-2 and IL-7. In addition, combinations of numerous members of the IL family as well as tyrosin kinase receptors flt3 (FL) and c-kit ligand (KL) are needed in different phases of NK cell development (Freud and Caligiuri 2006).

Mature NK cells: The mature NK cells distribute widely in lymphoid and non-lymphoid tissues. They are characterized by cell surface phenotype expressing CD56, which is a 140-kD isoform of neural cell adhesion molecule (NCAM), and by the natural cytotoxicity receptor NKp46. The latter is a member of the natural cytotoxicity receptor (NCR) family of NK-activating receptors. NK cells do not express CD3, a characteristic of T cells. The total population of mature human NK cells, defined as $CD3^{-}CD56^{+}NKp46^{+}$ cells, is not phenotypically and functionally homogenous. The $CD3^{-}CD56^{bright}NKp46^{bright}$ cells with high surface density expression of both CD56 and NKp46 produce large amounts of cytokines and chemokines very quickly upon activation. They include IFNγ, TNFα, GM-CSF, and IL-10 (Papamichail et al. 2004). However, they have very low or no ability to spontaneously kill tumor target cells. They reside mainly in lymph nodes and tonsils (Cheng et al. 2013). On the contrary, NK cells with low surface expression of CD56 and NKp46, called $CD3^{-}CD56^{dim}NKp46^{dim}$ cells, produce clearly less cytokines and chemokines. However, some are able to spontaneously lyse susceptible tumor target cells. $CD56^{bright}$ NK cells develop to $CD56^{dim}$ cells (Caligiuri 2008).

$CD56^{dim}$ cells express CD16, a low affinity crystallizable fragment gamma (Fcγ) receptor IIIA able to bind to the constant (Fc) region of immunoglobulin when expressed on a cell surface. These bindings cause a CD16 mediated activation of NK cells, resulting in perforin-dependent target cell lysis. This phenomenon is called antibody-dependent cellular cytotoxicity (ADCC). NK cells represent

approximately 10-15% of circulating lymphocytes (Freud and Caligiuri 2006); about 90% of them and of spleen NK cells are $CD56^{dim}CD16^{+}$ cells, the rest being $CD56^{bright}$ cells (Cheng et al. 2013).

NK cell activating and inhibiting factors: To assure that NK cells lyse only target cells with lost or deficient MHC I class antigen, a balance between activatory and inhibitory signals in the receptors with their corresponding ligands has been confirmed (Caligiuri 2008). Human NK cells have inhibitory receptors that recognize MHC class I molecules, including killer immunoglobulin-like receptors (KIRs) that bind to classical MHC class Ia ligands to human leukocyte antigen A, B, or C (HLA-A, B, C). However, some KIRs are activating receptors. The inhibitory CD94-NKG2 receptor binds the nonclassical MHC class Ib HLA-E. When contacting MHC class I, the inhibitory KIR sends a signal to the NK cell, which overrules all activating signals. Interestingly, KIRs with long cytoplasmic tails are inhibitory, whereas KIRs with short tails are activatory (Biassoni et al. 1996). Defective MHC class I expression may cause an attenuated NK cell response (Liao et al. 1991). Inhibitory receptors specific for self-MHC may give a greater responsiveness to NK cell (licensing) (Kim et al. 2005).

A large number of activating NK cell receptors are known (Lanier 2005). They induce various signalling cascades. Receptors associated with immunoreceptor tyrosine-based activation motif (ITAM) are divided into two groups. The first includes receptors like KIR2DS, KIR3DS, and NKG2C. The second consists of CD16, NKp30, and NKp46, and is expressed mostly on resting NK cells. A further group of activating receptors is not associated with ITAM. It includes receptors like NKG2D and NK, T- and B-cell antigens. NKG2A provides an inhibitory while NKG2C and NKG2E provide an activatory signal to the cell (Aramburu et al. 1990, Papamichail et al. 2004).

The activated NK cells may kill the target cells directly by releasing cytoplasmic granules containing perforin and granzymes, by death receptor-mediated apoptosis via TNF family, by secreting various effector molecules like IFNγ stimulating adaptive immunity, restricting tumor angiogenesis, producing and activating nitric oxide (NO) signalling, and through antibody-dependent cellular cytotoxicity by expressing (Cheng et al. 2013).

Normally, the inhibitory receptors like KIRs and NKG2A/B binding to various MHC-1 molecules, present in almost all cell types, inhibit NK cell activation and prevent NK cell-mediated killing. Under conditions in which cells lose or downregulate MHC-1 expression, NK cells lose inhibitory signalling. The so-called

"missing-self-recognition" is activated. When the target cells present pathogen-encoded molecules not expressed by the host or self-expressed proteins upregulated by transformation or infections, the "stress-induced self-recognition" is activated. IFNs or DC- or macrophage-derived cytokines like IL-2, IL-12, IL-18, and IL-15 may augment the activation and cytotoxicity of NK cells up to 200-fold (Cheng et al. 2013).

Ligands are molecules that produce a signal by binding to a target protein. Ligands for NKG2D, like nonclassical MHC class I chain-related gene A and B (MICA, MICB), are expressed on some tumor cells and on infected or stressed cells (Cerwenka and Lanier 2003). They are highly expressed on some tumors like melanomas and leukemic T cell lines, rendering these cells susceptible to NK cell mediated lysis. However, certain tumors downregulate NKG2D expression and function via TGFβ or soluble ligands (Moretta et al. 2006).

NK cell co-operation with other lymphocytes: NK cells might indirectly exert antitumor immunity by interacting reciprocally with DCs, macrophages, T cells, and endothelial cells. Activated NK cells produce IFNγ-inducing cytotoxic $CD8^+$ T cells (CTC) and $CD4^+$ T cells to develop into helper T cell 1 (Th1). NK cell-derived cytokines might also regulate antitumor antibody (Ab) production by B cells. NK cells also provide antigenic cellular debris for DCs, which present it to T cells, facilitating the generation of antigen-specific CTLs (Cheng et al. 2013).

NK cell cytotoxicity mechanisms: NK-cell-mediated cytotoxicity is the most important function of NK cells in immune-oncology (Caligiuri 2008, Vanherberghen et al. 2013). Vanherberghen et al. have conducted a detailed study of NK cell killing mechanisms at single-cell level. They used polyclonal NK cells from healthy donors' peripheral blood, isolated by negative magnetic beads ($CD3^-CD56^{dim}$). The main mechanism of target-cell killing was thereby shown to be exocytosis of granules containing granzymes and perforin. Granzymes induce apoptotic death, whereas perforin causes large membrane protrusions and kills the target cell quickly. The NK-target interaction comprises three phases, migration, conjugation, and attachment. They found five categories of NK cells: 1) NK cells that did not interact with target cells, 2) NK cells that did not kill, 3) NK cells that killed all target cells encountered, 4) exhausted NK cells, and 5) NK cells that kill stochastically. The exhaustion of NK cells was related to the depletion of perforin.

There was a significant heterogeneity of NK cells as to killing behavior. The mean conjugation time for nonlytic interactions was 81 min and for lytic events 65 min. In the length of attachment, no significant differences were found (128-154

min). The delivery of a lytic hit after conjugation occurred in most of the killing events in less than 20 min. The rate of target-cell death revealed two groups, fast and slow, depending on the amount of perforin released by the NK cells. The fast target-cell deaths demonstrated in 90% a rapid cell swelling and bursting, whereas the slow dying cells showed in 90% a blebbing consistent with apoptosis. The time from lytic hit to end of conjugation was eight times faster for the fast-acting NK cells (about 8 min vs. 60 min), demonstrating a correlation between the speed and strength of the NK cell response. During a single target interaction, NK cells can deliver from zero to four separate packages of lytic granules arriving at different times against a single target cell. The NK cells were able to form between zero and eight conjugates within 12 hours. About 50% of NK cells did not kill at all. About 5 % of the NK cells killed five or more target cells, totalling 26% of the number of kills. The least target cells were killed during a four-hour assay (Vanherberghen et al. 2013).

Dendritic cells and their development: Dendritic cells are bone marrow–derived phagocytic cells acting as antigen-presenting cells (APCs) in the innate immune system (Liu 2001). $CD34^+$ hematopoietic stem cells differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) and further into $CD11c^+CDa^+$ and $CD11c^-CDa^-$ immature dendritic cells (imDC). $CD11c^+CDa^+$DCs migrate into skin epidermis, becoming Langerhans cells, whereas $CD11c^-CDa^-$ DCs migrate into the skin dermis and other tissues. They are called interstitial DCs (IDCs). Further, they develop into monocyte precursors (pre-DC1) and plasmocytoid precursors (pre-DC2). Pre-DC1s differentiate into immature myeloid DCs (imDC1) by GM-CSF, IL-4, or phagocytosis. Pre-DC2s differentiate into imDC2s by IL-3 or via an innate immune response. DC1s produce IL-12 and induce T helper (Th1) and cytotoxic T cell (CTC) responses, whereas DC2s do not produce IL-2. They induce Th2 responses or generate IL-10 producing $CD8^+$ T suppressor cells. ImDCs migrate further into blood, lymphatics, lymph nodes, and nonlymphoid tissues functioning as the "police" of the immune system (Liu 2001, Steinman et al. 2000). They maintain peripheral self-tolerance by promoting naïve $CD4^+$ and $CD8^+$ T cells to differentiate into T regulatory or suppressor T cells. Following allogeneic transplantation, the donor or host DC might initiate graft rejection and graft versus host disease (Jonuleit et al. 2000, Liu 2001).

Dentritic cell functions: DCs sense pathogens directly or by cellular stress, leading to the release of cytokines and cellular proteins. DCs activate NK cells by

soluble factors and cell-to-cell contact. DC-derived IL-12 is a potent NK cell activator (Ferlazzo and Munz 2004). IFNα up-regulates the NKG2D ligands on DCs and activates resting NK cells in cell-to-cell contact. Thereby, DCs produce autocrine/paracrine IL-15 production, activating NK cells (Jinushi et al. 2003). DCs are able to connect the innate and adaptive immune systems (Liu 2001,Ferlazzo and Munz 2004). The NK cell–activating receptor NCRNKp30 stimulates NK cells to lyse immature or non-activated DCs from inflamed tissues. Thus, DC trophic pathogens are deprived of their host cells (Ferlazzo and Munz 2004).

Macrophages and their development: Macrophages (MΦ) are bone–marrow derived lymphoid cells of mononuclear phagocytic lineage (Murray et al. 2014). The diversity of macrophage functions and phenotypes according to their residing microenvironments has led to several different classifications, the newest one published in 2014 (Murray et al. 2014). Macrophages differentiate and polarize from human $CD14^+$ monocytes under the influence of the granulocyte-macrophage colony-stimulating factor (GM-CSF, GM-Φ) and M-CSF (M-Φ) GM-. MΦs present pro-inflammatory and M-MΦs anti-inflammatory phenotypes (Gonzalez-Dominguez et al. 2016). Classically, they are divided into M1 and M2 subgroups.

Macrophage functions: M1 MΦs are cytotoxic and produce inflammatory mediators like TNF, IL-1, and IL-12, as well as reactive oxygen and nitrogen species (ROS and RNS). They are important in host defense against intracellular pathogens and transformed cells. M2 MΦs express CD206, CD209, and CD163 and are associated with anti-inflammatory responses as well as angiogenesis, scavenging, and tissue remodeling (Gonzalez-Dominguez et al. 2016). Recently, specific markers for anti-inflammatory M-MΦs, CD163L1, and proinflammatory GM-MΦs, CLEC5A, were found (Gonzalez-Dominguez et al. 2016).

In tumor immune surveillance, macrophages have an immunoregulatory function (Pollard 2009). Activated macrophages can kill tumor cells and act as antigen-presenting cells to the cytotoxic T cells. Furthermore, they are able to eliminate metastases (Qian and Pollard 2010). They produce IL-10 inducing monocytes to express programmed death ligand (PD)-L1. In addition, MΦs can inhibit T cell responses. Macrophages have been shown to promote cancer initiation and malignant progression and to facilitate metastasizing (Qian and Pollard 2010).

In more than 80% of malignancies, there is a correlation between tumor infiltrating macrophage (TAM) density and poor patient prognosis, with a few exceptions (Kim et al. 2008, Qian and Pollard 2010). In addition, macrophages have a role in tissue development, including bone morphogenesis, ductal branching of mammary gland, neural networking, angiogenesis, and adipogenesis (Pollard 2009).

2.3.2 Some essential effector cells of the adaptive immune system in tumor immunosurveillance

T cells and their development: T cells derive from the common hematopoietic stem cells $CD34^+$ in bone marrow through a branched developmental system (Rothenberg et al. 2016). From bone marrow, they migrate to the thymus microenvironment via blood. The early intrathymic precursor (ETP) cells undergo a multistep specification and commitment development via activation of several stages by the Notch pathway using Notch ligand Delta-like 4 on the stroma of thymus with Notch1 molecules on ETP surfaces. ETPs go through several developmental steps, gaining a CD4 and CD8 double-positive stage and rearranged T cell receptor genes (TCR). Thus, they are committed T cells with T cell identity (Rothenberg et al. 2010, Rothenberg et al. 2016). They further develop into single positive $CD4^+$ and $CD8^+$ T cells. In the development of T cells, several interferons and tumor necrosis factors are necessary (Delves et al. 2006a). During intrathymic development, T cells reacting with self-antigens are deleted by negative selection, establishing a central tolerance of the T cells (Rothenberg et al. 2010).

T cell functions: T cells are able to directly kill foreign cells using the granzyme-perforin system. They are usually activated in lymph nodes after interaction with antigen-presenting cells, primarily dendritic cells (Melvold and Sticca 2007). $CD4^+$ T cells are dealing with the combination of peptides and MHC class II on the APC surface. If this is resulting in a tight binding to TCR, T cell activation and differentiation is initiated by binding several receptors on the APC and $CD4^+$ T cells. T cells secrete IL-2 as a proliferation signal and IL-2R receptor for the cytokine. Activated $CD4^+$ T cells leave the lymph nodes and recirculate in the body. $CD8^+$ T cells concentrate on combinations of peptides and MHC class I molecules on APCs' surfaces. Therefore, the binding of some receptors on the APC and T cell is necessary for further differentiation and production of IL-2 and

IL2R. Like $CD4^+$ T cells, the $CD8^+$ T cells circulate throughout the body (Melvold and Sticca 2007).

The activated $CD4^+$ T cells undergo a further differentiation into T helper 1 (Th1) or T helper 2 (Th2) cells, depending on the environment, cytokines, APCs, or organisms providing the antigen stimulus. Th1 $CD4^+$ T cells are mostly involved in cell-mediated responses and Th2 $CD4^+$ T cells in regulation of the antibody secretion by B cells. Activated $CD4^+$Th1 cells mediate delayed-type hypersensitivity (Melvold and Sticca 2007).

The activated $CD8^+$ T cells mediate CTL responses. They establish cell-to-cell contact with target cells presenting appropriate peptide-MHC I combinations. Both APC and non-APC cells might have those combinations on their surface. CTLs, like NK cells, are able to kill target cells directly or via apoptosis by exocytosis, using granules containing granzymes and perforin. Solid tumors often have infiltrating CTLs called tumor-infiltrating lymphocytes (TIL) that are able to kill corresponding tumor cells (Rosenberg and Restifo 2015, Rothenberg et al. 2016).

The amplitude and quality of T cell response is regulated by a balance between costimulatory and inhibitory signals. The inhibitory signals are called immune checkpoints. They maintain self-tolerance and prevent autoimmunity (Pardoll 2012). A powerful immune-checkpoint receptor is the CTL-associated antigen 4 (CTLA4) counteracting the activity of the T cell co-stimulatory receptor CD28. CD28 amplifies TCR signalling to activate T cells. CTLA4 competes with CD28 for identical ligands CD80 and CD86. CTLA4 outcompetes CD28 and delivers inhibitory signals to the T cell. In addition, it downmodulates helper T activity and enhances Treg immunosuppressive activity (Pardoll 2012).

Treg cells differentiate from $CD4^+$ cells via TGFβ, which is mainly produced by tumor cells. They are characterized by $CD4^+$ $CD25^+$ and the forkhead family transcription factor box 3 (Foxp3), and preferentially infiltrate the tumor microenvironment. High levels of Treg are associated with poor tumor prognosis; however, in some malignancies they are associated with a good prognosis (Curiel 2008).

Treg cells exhibit another checkpoint receptor, the programmed cell death protein 1 (PD-1). It limits the activity of T cells in peripheral tissues like tumors during an inflammation and limits autoimmunity. It has two ligands, PD-L1 (CD274) and PD-L2 (CD273). PD-1 engaged by the ligands inhibits T cell activation and influences T cell–APC contact. It diminishes the number and

suppressive activity of Treg. PD-1 is also expressed in B cells and NK cells (Pardoll 2012).

One part of the CTLs is able to develop a persistent population of antigen-experienced cells representing immunologic memory. For the development of persistent memory CD8+ T cells, the specific antigens, CD4+ T helper cells, and IL-7, IL-15, and IL-21 are necessary. This cell population is heterogeneous in phenotype, function, and protective capacity (Klebanoff et al. 2006).

Most T cells are αβT cells, having T cell receptors (TCRs) composed of two glycoprotein chains called α and β TCR chains. From 0.5 to 10% of T cells have TCR made up of one γ and one δ chain and are called γδT cells. They present characteristics of both innate and adaptive immunity. It is hypothesized that they predate the development of B and αβT cells. They are able to phagocytose, process antigens, present MHC class II, and act as APC. They have numerous, but not yet well understood, direct and indirect effects in the immunology (Wu et al. 2009).

Natural killer T cells (NKT) share characteristics of both NK and T cells. They develop in the thymus from the $CD34^+$ pool, express $CD3^+$, and fall into four different groups. Type I NKT cells, called invariant NKT (iNKT), are the most studied. iNKT cells respond rapidly to glycolipids presented on CD1d and α-galactosylceramide (αGalCer). iNKT cytotoxicity is mediated through the CD95-CD178 pathway. NK and iNKT cells are phenotypically and functionally similar. They are able to excrete cytokines like IL-4 and IL-13, discriminate self versus non-self, and contribute to tumor immunosurveillance (Chaplin 2010, Vivier et al. 2012).

B cells and their development: B cells, too, are derived from the common hematopoietic stem cell $CD34^+$. In the fetal phase, B cells differentiate in the liver, controlled by IL-7; after birth they differentiate in the bone marrow (Delves et al. 2006b). B lymphopoesis can be divided into at least four phases. During the differentiation, the rearrangement of immunoglobulin genes necessary to construct a functional B-cell antigen receptor (BCR) develops. The rearrangement and combinatorial association of gene segments ensures a vast repertoire of antibodies and B cell specificities (Hardy and Hayakawa 2001, Cain et al. 2009).

The self-reactive immature B cells are deleted by several mechanisms (Cain et al. 2009). B cells surviving this negative selection enter one of the mature B cell compartments, which include the mature follicular pool that recirculates through peripheral tissues and the fixed splenic marginal zone compartment. The mature follicular compartment is populated mainly by memory B cells, whereas a marginal

zone is provided for B cells capable of rapid but transient response (Gray et al. 1982, Cain et al. 2009). The final steps in B cell development occur in the spleen through type 1 transitional (T1) B cells and transitional 2 (T2) B cells. It is regulated by the B-cell activating factor of the TNF family (BAFF) (Cain et al. 2009), which also upregulates the expression of CD21 and CD23 B-associated receptors for complement and IgE production (Carsetti et al. 1995, Cain et al. 2009).

B cell functions: Activation of the B cells occurs in lymph nodes, where they can mingle with antigenic debris carried by dendritic cells or with activated T cells. Naïve B cells may find antigens able to bind to their surface receptors and send a signal to the nucleus for activation. In addition, another signal is needed from an activated Th2 cell via IL-4 as well as an interaction of membrane molecules of the two cells (Melvold and Sticca 2007). The activated B cell begins to proliferate and differentiate, mainly in the bone marrow and spleen. They express the antibody immunoglobulin M (IgM) on their surface. One part becomes plasma cells and another part memory B cells. Plasma cells lose surface IgM expression. Instead, they secrete soluble IgM until they "burn out" within some days. Memory B cells retain their IgM surface expression and migrate back into lymph nodes. When able to interact again with the same antigen and Th2 cells, they become reactivated. They produce antibodies that bind with increasing intensity and efficiency, a process known as affinity maturation. In addition, reactivated memory B cells can change from production of IgM to production of IgG, IgA, or IgE antibodies with the same antigenic specificity. Reactivated B memory cells might enter the plasma cell or the memory B cell pathway (Melvold and Sticca 2007).

2.4 Cancer immunotherapy

The existence of cancer in a body means that the immunosurveillance has failed. However, immunotherapeutic interventions may still be effective in fighting the cancer. Melvold and Sticca have presented a list of some principal methods used in tumor immunology (Melvold and Sticca 2007).

2.4.1 Possibilities of cancer immunotherapy

Active immunotherapy: The host's own immunosystem is actively stimulated by vaccination with purified tumor antigens, antigens from whole tumor cells, or tumor lysates, dendritic cell vaccines prepared by various techniques, DNA vaccines encoding for tumor antigens, and viral vector vaccines (Melvold and Sticca 2007). Tumour cell modifications are used to enhance host immunity by genetically engineering tumor cells to produce factors preventing tumor escape, like necessary but missing costimulating genes or cytokines to stimulate CTLs (Melvold and Sticca 2007).

Nonspecific active stimulation is used to activate or enhance certain aspects of the immune system. Extracts of mycobacterial materials induce an inflammatory reaction and activate macrophages. Antibodies and cytokines can be used in nonspecific immunostimulatory ways (Melvold and Sticca 2007).

Passive immunotherapy: Passive immunotherapy includes the transfer of antibodies and effector immune cells for the immunotherapy. Over 100 different monoclonal antibodies are developed for tumor-specific immunotherapy. They are used for antibody-mediated defense by, for example, complement activation, opsonization, phagocytosis, activation of intrinsic apoptosis pathways, inhibition of vascular growth factors, and inhibition of growth-signalling pathways (Melvold and Sticca 2007).

In adoptive cellular therapy, cultured immune cells with antitumor activity are transferred into a tumor-bearing host. Autologous tumor-infiltrating lymphocytes expanded in vitro with IL-2 and lymphokine-activated killer cells (LAK) derived from NK cells are used (Melvold and Sticca 2007).

In graft-versus-leukemia treatments of leukemias, a combination of T cells and stem cells are used to induce a graft-mediated immune reaction against the leukemia cells (Melvold and Sticca 2007).

However, the therapeutic results of the classical immunotherapies have been modest, even though Rosenberg et al. in the 1980s reported good results in melanoma and renal cell cancer patients using IL-2 activated tumor-infiltrating lymphocytes (TIL). In addition, the side effects have been remarkable (Rosenberg et al. 1984, Rosenberg and Restifo 2015). It is assumed that one of the reasons for suboptimal therapeutic results is the dual role of the immune system's ability to suppress but also enhance cancer initiation and promotion. In addition, tumor-

related chronic inflammation promotes an immunosuppressive tumor microenvironment (Shalapour and Karin 2015, Yang 2015). Tumor cells reprogram myeloid MDCCs and TAM cells to create an immunosuppressive microenvironment and promote angiogenesis and metastases. Furthermore, tumor cells promote the cancer immunoediting (Yang 2015).

Today, IFNα is approved for the postoperative adjuvant treatment of high-risk cutaneous melanoma. Mocellin et al. reviewed 10,499 patients in 18 controlled studies. According to meta-analysis adjuvant IFNα was associated with significantly improved disease-free and overall survival ($p = <0.00001$ and 0.003 respectively) (Mocellin et al. 2013).

Recently, new successes have been published regarding directing the balance of the immune system toward the elimination of cancer cells. This marks the beginning of a new era in cancer immunotherapy (Yang 2015). Inhibitory pathways (immune checkpoints) have been blocked using antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and PD-1 pathways of the T cells (Pardoll 2012).

CTLA-4 is able to down-regulate T cell activation pathways. Ipilimumab is a fully human monoclonal antibody (IgG1) capable of blocking CTLA-4 and thus promoting antitumor immunity (Pardoll 2012). Hodi et al. treated 125 patients with a diagnosis of unresectable previously treated melanoma in a randomized, double-blind phase 3 study with ipilimumab, with or without a glycoprotein 100 (gp100) peptide vaccine or gp100 alone. The median overall survival with ipilimumab alone was 10.1 months, with ipilimumab and gp100 10.0 months, and with gp100 alone 6.4 months ($p <0.001$) (Hodi et al. 2010).

A fully human IgG4 programmed death 1 (PD-1) immune-checkpoint inhibitor antibody (nivolumab) blocks interaction of the PD-1 receptor with its two ligands, programmed cell death protein ligands 1 and 2 (PD-L1 and PD-L2), preventing T cell suppression. Robert et al. conducted a phase 3 double-blind randomized and controlled study using nivolumab to treat 418 patients with metastatic melanomas without BRAF mutation and compared the treatment results with dacarbazine treatment. The overall survival and the objective response rate of the nivolumab group was significantly higher than those of the dacarbazine group ($p = 0.001$ and 0.001 respectively) (Robert et al. 2015a). Using pembrolizumab, another anti-PD-1 antibody, Robert et al. achieved comparable results to nivolumab in patients with advanced melanoma (Robert et al. 2015b).

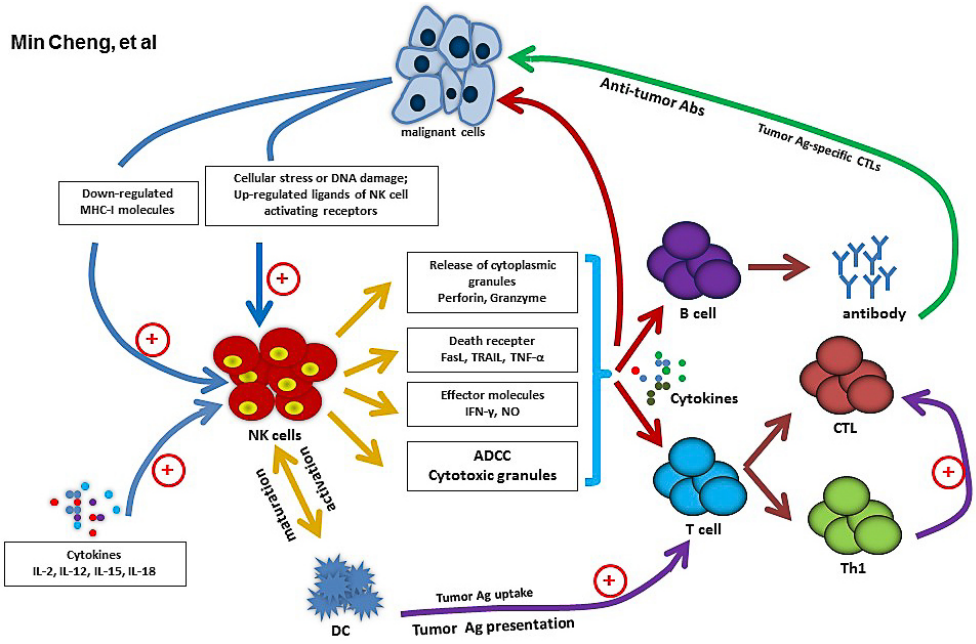
Larkin et al. published results of a randomized, double-blind phase 3 study in 2015. They combined nivolumab and ipilimumab in treating 945 untreated melanoma patients and compared the progression-free survival and overall survival with patients treated with nivolumab alone or ipilimumab alone. The median progression-free survival was 11.5 months with the nivolumab plus ipilimumab, 2.9 months with ipilimumab, and 6.9 months with nivolumab. (Larkin et al. 2015).

Monoclonal antibodies have been developed for cancer therapy. Anti-CD20 monoclonal antibody rituximab is in daily use in treating non-Hodgin's lymphoma patients (Iannello and Ahmad 2005) and anti-HER2 monoclonal antibody trastuzumab in breast- and gastric-cancer patients (Alderson and Sondel 2011). Loi et al. showed for the first time in triple-negative breast cancer patients overexpressing HER2 an association between higher levels of tumor-infiltrating lymphocytes and an increased trastuzumab benefit (Loi et al. 2014). Mittal et al. demonstrated in a mouse breast-cancer model that trastuzumab therapy required IL-21 signaling in $CD8^+$ T cells. Administering recombinant IL-21 in combination with trastuzumab was therapeutic against primary and metastatic breast cancer (Mittal et al. 2016).

2.4.2 Natural killer cells in cancer immunotherapy

It is shown that natural killer cells can eradicate tumor cells and are important in tumor immunosurveillance (Smyth et al. 2002, Cheng et al. 2013) (Figure 2).

Figure 2. NK cells in tumor immunosurveillance.



The diagram shows the potential roles of NK cells in tumor immunosurveillance. NK cells initially recognize the tumor cells via stress or danger signals. Activated NK cells directly kill target tumor cells through at least four mechanisms: cytoplasmic granule release, death receptor–induced apoptosis, effector molecule production, or ADCC. Additionally, NK cells act as regulatory cells when reciprocally interacting with DCs to improve their antigen uptake and presentation, facilitating the generation of antigen-specific CTL responses. Also, by producing cytokines such as IFN-γ, activated NK cells induce CD81 T cells to become CTLs. Activated NK cells can also promote differentiation of CD41 T cells toward a Th1 response and promote CTL differentiation. Cytokines produced by NK cells might also regulate antitumor Ab production by B cells. Ab, antibody; ADCC, antibody-dependent cellular cytotoxicity; CTL, cytotoxic T lymphocyte; DC, dendritic cell; IFN, interferon; NK, natural killer. (Courtesy of Chen et al. 2013)

High levels of tumor infiltrating NK cells (TINKs) are associated with favorable tumor outcome. Ishigami et al. used CD57 immunoglobulin to stain immunoihistochemically NK cells in specimens of gastrectomized gastric carcinoma patients. A high level of intratumoral CD57 NK infiltration was associated with fewer lymph node metastases, less lymphatic invasion, venous invasion, and lower clinical stage of disease. Furthermore, the 5-year survival rate of patients with high rate of NK invasion was significantly better than that of patients with a low level of NK infiltration ($p < 0.01$) (Ishigami et al. 2000). Also, Villegas et al. used monoclonal antibody CD57 for staining and received similar results in patients with primary squamous cell lung carcinoma (Villegas et al. 2002). Conversely, Rathore et al. studied patients with infiltrating ductal carcinoma of the breast and used antibodies specific for immune staining of intratumoral CD56 NK cells. They found that high intratumoral CD56+NK-TILs counts were associated with lower survival than in patients with low CD56 intratumoral count ($p < 0.05$) (Rathore et al. 2014).

Obtaining a sufficient number and purity of functional NK cells is a key factor of NK cell–based immunotherapy (Arai and Klingemann 2005). NK cells may be produced from peripheral blood, cord blood, bone marrow, and embryonic stem cells (Cheng et al. 2013). Multipotent $CD34^+$ hematopoietic stem cells allow in vitro production of different kinds of NK cells, some with high NKG2D levels and NK cytotoxicity receptors (Spanholtz et al. 2010). In addition, bone marrow derived $CD34^+$ cells, processed in vitro by IL-2 and allogeneic feeder cell layers and other hematopoietic growth factors result in strong cytotoxicity and production of several effector molecules for natural cytotoxicity (Miller et al. 1994).

NK cells isolated from peripheral blood mononuclear cells (PBMCs) have been successfully expanded in vitro for clinical immunotherapy (Cheng et al. 2013). The expansion can be augmented by using feeder cells like genetically modified K-562 cells, irradiated autologous cells, IL-2, IL-15, and anti-CD3 antibodies (Berg et al. 2009). Currently, NK cells are expanded in large-scale, clinical-grade and feeder-free automated bioreactors (Sutlu et al. 2010).

Cheng et al. and Mc Dowell et al. have set up comprehensive lists of preclinical and clinical studies of adult and pediatric cancer patients treated with NK cell-associated therapies (Cheng et al. 2013, McDowell et al. 2015).

In early studies, the goal was to improve antitumor activity and proliferation of autologous NK cells using systemic administration of cytokines like IL-2, IL-12, IL-15, IL-18, IL-21, and type I interferons. Upon cytokine stimulation, NK cells

become lymphokine-activated killer (LAK) cells with enhanced cytotoxicity against tumor cells. However, the clinical success has been limited due to toxicity of systemic cytokine administration and cytokine-induced NK cell apoptosis (Zamai et al. 2007). However, combining IL-2 and IFNα with GM-CSF has been shown to be effective (Sutlu et al. 2010).

Adoptive therapy with NK cells has been practiced. Promising clinical results have been achieved in hematopoietic malignancies. In the Haploidentical Natural Killer Cell Transplantation for Acute Myeloid Leukemia (NKAML) study, Rubnitz et al. treated pediatric patients with acute myeloid leukemia (AML) in remission. Patients received immunosuppressive treatment followed by killer immunoglobulin-like receptor–human leukocyte antigen (KIR-HKA) mismatched NK cells and IL-2. After a median follow-up time of 964 days, all 21 patients remained in remission (Rubnitz et al. 2010). Miller et al. treated 19 poor-prognosis adult AML patients using high intensity immunosuppressive chemotherapy followed by activated, haploidentical donor NK cell infusions. Five out of 19 patients had a complete hematologic remission (Miller et al. 2005).

Allogeneic hematopoietic stem cell transplant (HSCT) techniques have shown some promising clinical results. However, there still are difficulties in finding matching donors and avoiding graft versus host disease (GVHD) (McDowell et al. 2015).

$CD16^+$ NK cells have activating FcRγIIIa receptors on the cell surface and are able to recognize antibody-coated target cells and mediate ADCC cytotoxicity. In some instances, inhibitory signals predominate over activating signals. Therefore, NK cell-mediated cytotoxicity does not occur. When there are specific antibodies bound to the surface of tumor cells, the activating signals may predominate via FcRγIIIa and ADCC may occur (Papamichail et al. 2004, Caligiuri 2008).

Several attempts have been made to alter the antibody structure of tumor cells to have a stronger NK tumor cell recognition and immunologic response. Tumor antigens may be altered using class switching and point mutations and producing specific monoclonal antibodies (mAbs) against tumor cells (Iannello and Ahmad 2005, Alderson and Sondel 2011). Monoclonal antibodies have been conjugated to cytokines by fusing the human gene for the cytokine to the mAb gene. Coadministration of cytokines, TLR agonists, IL-12, and IL-21 might potentiate ADCC (Cheng et al. 2013).

Genetic modifications of NK cells have been conducted using gene transfer with cytokine transgenes. Genes have been altered in order to overexpress

activating receptors, silencing inhibitory receptors or retargeting NK cells via chimeric receptors, and thus to modulate and enhance NK cell-tumor interaction (Nagashima et al. 1998). Numerous phase 1 to 2 studies have been conducted or are ongoing to treat solid and hematological malignancies in different settings. The results have been conflicting. Despite the recent advances, there are principal difficulties to overcome, including significant tumor heterogeneity in genetic and epigenetic properties and cancer stem cells capable of repopulating tumors (Grossenbacher et al. 2016).

New techniques are being introduced, including chimeric antigen receptors (CARs), in which two components are linked together by a transmembrane domain. One component is a genetically engineered "hybrid" receptor comprising an extracellular single-chain antibody fragment recognizing the tumor-associated antigen. The other component is an intracellular signal moiety. Typically, CARs are introduced into effector NK cells and integrated into genetic sequence using g retroviruses. The incorporation of tumor-specific CARs into NK cells enables them to directly bind a specific antigen on tumor cells. Studies are ongoing (Imai et al. 2005).

2.5 Temperature and cancer treatment

2.5.1 Hypothermia

Hypothermia is defined as a body core temperature below 35.0°C (Brown et al. 2012). Primary hypothermia occurs when heat production in an otherwise healthy person is overcome by the stress of excessive cold. Secondary hypothermia can occur in persons with any of a variety of illnesses related to impaired thermoregulation, peripheral failure, or endocrinologic failure, or with insufficient energy or neuromuscular impairments. Increased heat loss may also induce hypothermia, including heat loss due to anesthesia and perioperative hypothermia or cold infusions in brain and spinal-cord injury treatments (Brown et al. 2012).

The severity of hypothermia can be estimated clinically by vital signs using the Swiss staging system (HT I-IV). In HT I, the patient is conscious and shivering with a core temperature from 35°C to 32°C. In HT II, the consciousness is impaired, the patient shivering, and the core temperature <32°C to 28°C. In HT

III, the patient is unconscious, not shivering, with vital signs present and a core temperature of <28°C to 24°C. In HT IV, no vital signs are detectable, and the core temperature is <28°C. The traditional staging system categorized hypothermia as mild, moderate, severe, or profound (Danzl and Pozos 1994). The Swiss staging system is preferred if it is not possible to determine an accurate core temperature (Durrer et al. 2003).

Physiological responses to cold exposure: The initial response of the body to hypothermia is to maintain a normal core temperature at approximately 37°C (Brown et al. 2012). The body can increase thermogenesis by shivering and amplifying peripheral vasoconstriction (Castellani et al. 2002). Shivering is an involuntary, repeated, rhythmic muscle contraction, the same than the muscular contractions experienced during exercise. Most of the metabolic heat production of shivering aids in maintaining the core temperature. The increased metabolic heat production caused by shivering is two to five times greater than resting levels. There is virtually no possibility of increasing metabolic heat production through nonshivering thermogenesis (Castellani et al. 2002).

In addition, the body limits heat loss by peripheral vasoconstriction. Vasoconstriction is controlled by alpha-adrenergic receptors. The reduced peripheral blood flow decreases heat flow between the body core and shell, thereby increasing effective insulation (Frank et al. 1996). Blood is redistributed to the core, increasing the cord stroke volume. Thus, the cardiac output increases, supporting higher metabolic heat production. The redistribution of blood flow and increase of cardiac output is shown to affect components of the immune system during both exercise and cold exposure (Castellani et al. 2002).

Cold exposure increases plasma norepinephrine concentration, which is a marker of the sympathetic nervous system (SNS). SNS, in turn, mediates and modulates immune functions (Castellani et al. 2002). Pedersen et al. showed in a tumor-bearing mice model that exercise links exercise, epinephrine, and IL-6 to NK cell mobilization and activation (Pedersen et al. 2016). Lackovic et al. demonstrated that volunteers exposed to 4°C for 30 min showed on average 0.45°C decrease of body temperature and an increase of norepinephrine and NK activity (Lackovic et al. 1988). Brenner et al. exposed seven healthy men for two hours at 5°C in a climatic chamber. The subjects were pre-treated for one hour in a water bath at 35°C (control) or at 38°C, then subjected to exercises at 55% of peak oxygen at 18°C as well as 35°C. Total NK cell activity increased significantly within cold exposure. In addition, other pre-treatments augmented NK activity (Brenner

et al. 1999). Cold exposure can differentially modulate cytokine production. The changes are linked to enhanced catecholamine secretion associated with cold exposure (Rhind et al. 2001). In addition, Th1 cytokines (IL-2, IFNγ) promoting killer $CD8^+$ cells are downregulated by cold exposure in the absence of a strong Th2 response. The cytotoxic T cells may be less effective after three to six hours cold exposure (Castellani et al. 2002).

Temperature and the tumor microenvironment: The tumor microenvironment harbors several immunosuppressive and tumor-promoting factors (Grivennikov et al. 2010, Gajewski et al. 2013). During tumor progression, disordered vascularization develops, which leads to ischemia (Jain 2005). Thus, the tumors might become colder than the microenvironment (Du 2013). Du et al. studied the role of hypothermic microenvironment in tumor immune subversion in mice models in vitro and in vivo. Lowering the temperature from 37°C to 31–34°C, lymphocyte proliferation in spleen decreased in a temperature-dependent manner. The cytolytic cell combination of $CD4^+$Th1 and cytotoxic $CD8^+$ cell, expressing IFNγ and IL-2, decreased significantly, whereas the $CD4^+$ Th2 cells with IL-4 and IL-10 expression substantially increased. The whole body hypothermia at <34°C showed an increase of inhibitory $CD4^+CD25^+FOXP3^+$Treg cells and $CD4^+$Th2 cells. Concomitantly, the number of cytotoxic $CD8^+$ and $CD4^+$Th1 cells decreased. The local tumor temperature downregulated the tumor immune rejection. Furthermore, local hypothermia induced local microcirculation dysfunction and increased intratumor Treg population as well as TGF-β1 in the tumor tissue. In addition, local hypothermia increased lung metastasis. The authors proposed a novel model of tumor immune subversion termed "the cold immunosuppressive effect" (Du et al. 2013).

The imbalance between Th1 and Th2 is connected to the pathogenesis of allergic and autoimmune disorders as well as to the immunosurveillance of tumors. Arai et al. used stimulated PBMC in vitro at 37°C and 30°C, and studied Th1 cytokine IFNγ and Th2 cytokines IL-4 and IL-5 as well as IL-10 and IL-12 production. At 30°C, IFNγ, IL-12 mRNA, and IL-12 protein production were upregulated due to the augmented nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). At the same time, IL-5 and IL-10 production was downregulated. They concluded that hypothermia modifies the pattern of cytokine gene expression (Arai et al. 2008).

Hypothermic therapies: Hypothermia has been used in treating cerebral ischemic stroke (Ceulemans et al. 2010) and in clinical settings in cardiac arrest in

order to reduce risk affecting the neurological outcome of patients who initially survive a cardiac arrest (Hachimi-Idrissi et al. 2014) .

2.5.2 Hyperthermia

Cellular effects of hyperthermia: Hyperthermia is defined in humans as a temperature higher than 37.5°C or 38.3°C, depending on the publication, fever as temperatures ≥38.3°C, and high fever as temperatures ≥39.5°C (Axelrod and Diringer 2008, Laupland 2009). Thermal ablation occurs in temperatures from 48°C to 60°C (Lepock et al. 1993).

It is known that hyperthermia in the range of 40°–47°C has numerous effects on living cells in a time- and dose-dependent manner (Roti Roti 2008). The thermal dose is a combination of heating time and temperature. The cell survival curves of clonogenic cells for temperatures of 42.5°C and below show a shoulder, an exponential portion followed by a plateau in the cell killing. Curves in the range of 43–47°C also demonstrate a shoulder but are followed by an exponential reduction (Sapareto et al. 1978). The survival curves can be described using mathematical models analogous to survival curves from radiation, for example the single-hit multitarget and the linear quadratic models (Dewey et al. 1977).

Hyperthermia causes a large number of macromolecular changes, depending on temperature. They, in turn, can lead to cell death either by hyperthermia-induced apoptosis or by mitotic catastrophe secondary to alterations in the DNA supporting proteins. Most cell lines die by a combination of these death processes (Roti Roti 2008). In cells, hyperthermia might cause unfolding of proteins and aggregate them. Proteins within the nucleus are critical to managing the proper transcription, replication, and repair of DNA. Alterations in these proteins result in chromosome aberrations, genomic instability, improper chromosome segregation, and cell death (Wong et al. 1993). The thermal effects on proteins in the hyperthermic range (40°C–47°C) might be reversible in the presence of molecular chaperones, such as heat shock protein 70 (HSP70). However, in thermal ablation temperatures (48°C–60°C), severe protein denaturation occurs and the protein changes are irreversible (Lepock et al. 1993).

It is deduced that DNA damages are not due to direct heat-induced DNA alterations, but are secondary to heat effects on proteins involved in DNA

replication, chromosome segregation, and DNA repair. Hyperthermia-induced γH2AX foci are markers for DNA double-strand breaks (Olive 2004).

The most vulnerable phase of the cell cycle is the S-phase. At temperatures ≤42.5°C, the major component of cell killing is observed in the S-phase due to potentially lethal lesions. If the thermal lesion can be repaired, it can be completely reversed. DNA synthesis is delayed long enough for the repair to be completed. However, the heat shock causes a disruption of the timing and coordination of DNA replication as cells progress through the S-phase (Roti Roti 2008).

When the thermal treatment is given several times with pauses between them, repairs can be observed in the normal and malignant cells. The repairs occur during the first hours between the treatments, reaching the maximum by about 24 hours. For prolonged heating at temperatures below 43.5°C, a decrease in the slope of the cell survival curve can be observed after about three to four hours of heating. If cells or tissues are heated for a short time followed by a return to normal temperatures, a transient reduction in sensitivity to a second heat treatment follows (Field and Bleehen 1979).

Effects of hyperthermia on immunological system: Fever is a response to infections. In the induction and maintenance of fever, the body temperature is regulated by the innate immune system and neuronal events (Evans et al. 2015). Indications of infection are issued via the binding of pathogen-associated molecular patterns to pathogen recognition receptors (PRRs), such as toll-like receptors (TLRs), on innate immune cells, including macrophages, neutrophils, and dendritic cells. For example, a lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall, is produced by hematopoietic cells following LPS-mediated activation of TLR4. It travels through the blood/brain barrier and initiates fever via prostaglandin E2 (PGE2) binding to EP3 prostaglandin receptors expressed by thermoregulatory neurons located in the hypothalamus. PGE2 is considered to be a major pyrogenic mediator of fever (Matsumura et al. 1998). Body temperature is elevated by the release of norepinephrine, which, in turn, increases thermogenesis in brown adipose tissue and induces vasoconstriction in the extremities to reduce passive heat loss (Hasday et al. 2014). Furthermore, the neurotransmitter acetylcholine stimulates the musculature to convert stored chemical energy into thermal energy and thus increase overall metabolic rates (Hasday et al. 2014).

The LPS-induced TLR4 augments the synthesis of pyrogenic cytokines like IL-1, TNF, and IL-6 at the site of infection as well as within the brain. IL-6 is

important in inducing and sustaining fever (Hasday et al. 2014). In the brain, the cytokines produced augment the synthesis of cyclooxygenase 2 (COX2), which is essential to produce PGF2 (Cao et al. 1996).

Fever enhances the immune protective mechanisms during infection (Evans et al. 2015). The same pyrogenic cytokines produced during the induction of fever also operate locally to regulate immunity within infected tissues (Soehnlein and Lindbom 2010). Innate immune cells respond within hours to pathogens until a peak adaptive immune response is generated, normally one week later. Macrophages and DCs bridge the gap between innate and adoptive immunity. The co-localization of DCs and T cells near high endothelial venules (HEVs) of lymph nodes is essential. HEVs are major portals for entry of blood-borne lymphocytes (Girard et al. 2012).

Effects of febrile temperatures on innate immunity: Thermal stress increases numbers of circulating neutrophils and recruitment to local sites of infection as well as to tumors regulated by granulocyte-colony stimulating factor (G-CSF) (Ostberg et al. 2005). Hematopoietic stem cells within the bone marrow are augmented, depending upon enhanced production of IL-17, IL-1β, and IL-1α (Evans et al. 2015). Heat-induced neutrophil recruitment in the tissues, for example in the lungs, is under the control of the heat-inducible transcription factor heat shock factor 1 (HSF1). This, in turn, regulates HSP and IL-18; thereby, the endothelial barrier integrity is decreased (Evans et al. 2015). However, temperatures above the normal febrile range impair neutrophil accumulation and function (Ostberg and Repasky 2000).

NK cell cytotoxicity and recruitment to tumor sites is increased by fever-range hyperthermia in vivo (Zanker and Lange 1982, Kappel M et al. 1991), whereas in vitro it is reduced in a heating-time- and temperature-dependent manner (Azocar J. et al. 1982, Nurmi et al. 1982, Onsrud 1988). The enhancement depends on heat-induced upregulation of the NKG2D ligand MICA on tumor cells and on the clustering of NKG2D receptors on the surface of NK cells (Ostberg et al. 2007). Furthermore, heat decreases MHC class I expression on tumor cells and simultaneously increases HSP70 production. Both of these responses enhance the cytotoxicity of NK cells (Multhoff et al. 1995a).

Whole body heating at fever-range temperatures in mice improves bacterial clearance, mostly by macrophages of the liver and increased serum concentrations of TNF, IL-1, and IL-6. Hyperthermia induces the upregulation of HSP70, which is required for enhancing the expression of nitrite oxide. HSP70 can be released

from cells into the extracellular environment, where it acts as a damage-associated molecular pattern (DAMP) to stimulate macrophages and DCs (Gupta et al. 2013). Furthermore, extracellular HSPs provide danger signals to enhance inflammation, whereas intracellular HSP is involved in suppression of inflammatory signals (Ito et al. 2001, Schmitt et al. 2007).

Elevated temperatures also enhance the phagocytic potential of DCs. In addition, DC expression of MHC class I and class II molecules and co-stimulatory molecules like CD80 and CD86 are increased. Moreover, the secretion of Th1 cell-polarizing cytokines IL-12 and TNF are augmented (Basu et al. 2001). Febrile-range temperatures augment the migration of antigen-presenting cells (APCs) to draining lymph nodes, which involve the increased responsiveness of CC-chemokine receptor 7 (CCRT7) to its ligands. CCRT7 axis regulates the optimal position of DCs in lymphoid organs at sites where they can present antigens to lymphocytes upon their arrival via HEVs (Tal et al. 2011). Fever-range thermal stress augments the ability of DCs to stimulate T cells by heat-induced upregulation of MHC class I and HSP70 in mature DCs. In addition, the thermal stress enhances proliferation of naïve $CD4^+$ cells, and promotes their differentiation toward a Th1 phenotype (Evans et al. 2015).

Effects of hyperthermia on adaptive immunity: A very important factor for generating adaptive immunity is the high rate of lymphocyte trafficking through lymphoid organs (Evans et al. 2015). This increases the probability that even rare antigen-specific T cells will receive activating signals from DCs (Blattman et al. 2002). The entry of blood-borne T and B cells into lymphoid organs occurs preferentially at HEVs. Four adhesion cascades are involved: 1) L-selectin and/or $\alpha4\beta7$ integrin, 2) CCl21-dependent activation of CCR7 on adherent lymphocytes, 3) lymphocyte function-associated antigen 1 (LFA-1)-mediated arrest via binding to its endothelial counter-receptors, which are intracellular adhesion molecule-1 (ICAM1) and ICAM2, and 4) LFA1-ICAM1-2-directed transendothelial migration (Evans et al. 2015). Fever-range temperatures augment the trafficking by complex mechanisms (Evans et al. 2015).

Antigen-driven $CD8^+$ T cell activation by APCs under thermal treatment directs $CD8^+$ T cells' differentiation to an effector phenotype. Thereby, a pronounced L-selectin downregulation enhanced cytotoxic function and increased production of IFNγ is observed (Mace et al. 2011). The mechanisms underlying thermal regulation of trafficking are controlled by IL-6, which also controls both lymphocyte and endothelial adhesion. For thermal response, a soluble form of the

IL-6 receptor, α subunit (sIL-6Rα), is necessary (Chen et al. 2004). The recruitment of cytotoxic $CD8^+$ cells across tumor-associated vessels in fever-range hyperthermia is mediated by IL-6 trans-signalling. Direct exposure of T cells to fever-range hyperthermia increases their proliferation (Fisher et al. 2011).

Hyperthermia in NK cell mediated anti-tumor responses: When treating cancer patients with hyperthermia at temperatures above 40°C, both enhancing and inhibitory effects on NK cell cytotoxicity against tumor cells have been reported (Dayanc et al. 2008). In the early phase of NK cell activation, pro-inflammatory cytokines in the microenvironment regulate NK cell activation. TNFα recruits NK cells to the inflammatory sites (Dayanc et al. 2008), where pro-inflammatory cytokines activate them. Pro-inflammatory cytokines like TNF-α and IFN-α/β are also pyrogenic. They stimulate an increase in body temperature. The induction of NK cell cytotoxicity is regulated by a complex mechanism of balance between activating receptors, e.g. NKG2D, NCR, NKp30, NKp44, and several inhibitory receptors, i.e. KIR and CD94-NKG2 heterodimers. The NK cell activation is regulated by the signalling strength between activating and inhibitory receptors (Smyth et al. 2001). NK cells form an immunological synapse with the target cell. This immunological synapse is thermally sensitive (Smyth et al. 2001).

Dayanc et al. have set up a comprehensive list of hyperthermic treatments in humans and animals (Dayanc et al. 2008). Clinical studies reveal an enhancement of NK mediated cytotoxicity upon hyperthermia treatment of either cancer patients or healthy subjects. Dayanc's own group observed an immediate increase of NK cell numbers in peripheral blood after six hours of whole body hyperthermia treatment at 39.5°C–40°C (Kraybill et al. 2002). Ostberg et al. observed that heat shock temperatures (42°C, one hour) on tumor cells significantly enhanced NK cell killing of KM123 colon carcinoma cells, but not Colo205 cells (Ostberg et al. 2007). In addition, hyperthermia was seen to inhibit NK cell cytotoxicity in vitro. NK cell cytotoxicity decreased at 40°C in vitro or at 39°C for 18 hours (Azocar et al. 1982, Nurmi et al. 1982, Dinarello et al. 1986). The conflicting results might relate to the variety of heating temperatures and times (Dayanc et al. 2008). There are also conflicting reports of NK cell distribution in peripheral blood, tumors, and sites of inflammation in response to temperature changes. In animal models, an increased number of NK cells were found to infiltrate the tumor bed (Kubes et al. 2008).

NK cells secrete IFNγ. Elevated temperatures (39.4°C) greatly enhance the anti-proliferative function of IFNγ on tumor cells (Fleischmann et al. 1986). Thermal

stress on the activating receptor NKG2D causes a clustering of the receptors without changing the NKG2D surface expression. NKG2D ligand MICA also clusters at the NK cell synapse in lipid rafts. The NK cell activation state in the peripheral blood (resting NK cells) might be fundamentally different from its activation state in culture conditions with IL-2 (activated NK cells). Sensitivity to thermal stress might be affected by the activation state. The cytotoxicity of freshly isolated NK cells is enhanced by incubation at 39.5°C, while IL-2 activated NK cells fail to demonstrate a thermally enhanced cytotoxicity (Ostberg et al. 2007). IL-2 activated NK cells show enhanced NKG2D clustering. Thus, the clustering could not be further enhanced with hyperthermia (Dayanc et al. 2008).

Hyperthermia at 45°C for 30 minutes has been shown to downregulate MHC class I molecules on melanoma cells (Blom et al. 1997), whereas in rat glioma cells, hyperthermia (43°C for one hour) increased MHC class I presentation on the cell surface, leading to tumor suppression in vitro (Ito et al. 2001). MHC class I expression was unchanged on K562 cells heated at 41.8°C (Multhoff et al. 1995a).

On tumor cells, heat shock induces the synthesis of heat-inducible proteins, so-called heat shock proteins (HSPs), including several subgroups. They play an important role in the activation of NK cells and might mediate the NK cell response to thermal stress. HSP70 expression was upregulated by heat shock (42.5°C for 90 min) on osteosarcoma, chondrosarcoma, and melanoma cells leading to increased susceptibility to NK cells (Multhoff et al. 1995). An HSP70-derived peptide and HSP70 positive tumor exosomes induce chemotaxis and cytotoxic activity of purified NK cells (Gastpar et al. 2005). The stress-inducible, non-classical MHC class I ligand A (MICA) is recognized by the NKG2D activating receptor on NK cells. The expression of the gene for MICA is enhanced with heat shock in some cell lines (Dayanc et al. 2008).

The available information concerning the thermal enhancement of NK cells for clinical use is still insufficient and conflicting. Several phenomena need to be investigated, including other stimulatory receptors and corresponding ligands, inhibitory receptors, and their ligands (Dayanc et al. 2008).

Hyperthermic treatment of cancer: Hyperthermia was one of the first cancer therapies (Overgaard 1985). Today, hyperthermia is a highly sophisticated treatment modality in oncology. It can be applied as a systemic therapy to the whole body, regionally, or locally and in an ablative manner. Historically, hyperthermic cancer therapy was linked to the anti-cancer benefits of high fever (Busch 1866, Overgaard 1985). Modern studies are focused primarily on the direct

cytotoxic effects of high temperatures in tumor cells and intra-tumoral vasculature. The indirect effects of hyperthermia on blood flow and tumor microenvironment are also investigated (Dayanc et al. 2008), including tumor re-oxygenation (Dewhirst et al. 2005). Studies have shown the potential of hyperthermia as an adjuvant to the immune system in creating stronger therapeutic anti-tumor responses. A major question is the optimal temperature range and treatment duration in treating cancer patients. Important topics are also the questions of effective heat delivery and verification systems (Mallory et al. 2016). In clinical situations, it is known that cancer cells and tumor tissue may be more heat sensitive than healthy cells due to tumor vasculature (Mallory et al. 2016).

There are numerous ways to induce hyperthermia in cancer patients. It can be applied using conduction, convection, or radiation techniques, by chemical, mechanical, bioactive, or electromagnetic means non-invasively, semi-invasively, or invasively (Szasz 2011). Today, nanoparticles are also used in hyperthermic treatments. Furthermore, liposomes carrying drugs that release their contents in response to heating are used (Wessalowski et al. 1998, Mallory et al. 2016). The temperatures used in whole body treatments vary from 38°C to 42°C and in the loco-regional therapies from 40 to 45°C. To reach the therapeutic temperature, times of 30–180 min are necessary and the treatment times are usually 120–720 min. The times for local and regional therapies are usually shorter (Szasz 2011). Online 3D hyperthermic planning systems are used for consistent heat delivery. It is possible to monitor the treatment temperature using magnetic resonance devices (MRIs). Quality assurance guidelines and recommendations for clinical practice have been published (Colombo et al. 2011, Mallory et al. 2016).

Today, hyperthermia is seldom used on its own in cancer treatment. Zwischenberger et al. used it in treating metastatic lung cancer. They administered extracorporeal veno-venous perfusion-induced systemic hyperthermia to elevate the core temperature from 42.0°C to 42.5°C for two hours. Patients had advanced non–small cell lung carcinomas (stage III–IV). The median survival time for these patients was 450 days; for controls it was 96 days ($p < 0.05$). However, the number of treated patients was small (Zwischenberger et al. 2004).

Gabriele et al. treated heavily pre-treated patients with different recurrent or metastatic cancers using a multifrequency device to create localized hyperthermia. The treatment protocol consisted of heating for 45 minutes at the intratumoral temperature of at least 42°C, twice a week for a total of six to 10 sessions. Complete responses were obtained in 16.6% and partial responses in 23.4% of

patients. Adenocarcinoma was the most heat-sensitive histologic cancer, with 40% of CR and 33.3% PR responses, whereas squamous cell carcinomas showed 7.7% of CR and 20.6% PR responses. The authors reported minimal side effects and complications (Gabriele et al. 1990).

Hyperthermia has been successfully combined with surgery and chemotherapy. Neuwirth et al. have summarized results of cytoreductive surgery (CRS) followed by hyperthermic intraperitoneal chemotherapy (HIPEC) in selected patients with peritoneal carcinomatosis of different malignancies The combination therapy has significantly improved disease-free and overall survival and reduced the frequency of recurrences (Neuwirth et al. 2016).

2.6 Combined effects of radiation, immunology, and hyperthermia

Radiation effects on NK cells: Studies showed early on that radiation inhibits NK cell activity in a dose-dependent manner (Schacter et al. 1985). Radiation sensitivity is controlled by a diallelic gene on the X chromosome (Brovall and Schacter 1981). Irradiation doses up to 10 Gy do not produce significant morphological alterations in NK cells, whereas higher doses lead to chromatin decondensation or nuclear pyknosis. Irradiation damages the NK cell functions at the postbinding level and mainly at the lytic functions. The cells are able to bind to tumor cells, but they are not activated and they are unable to release the cytotoxic enzymes from the granules (Zarcone et al. 1989). Low doses of irradiation up to 10 Gy increase NK cell cytotoxicity, while higher doses decrease the activity in a dose-dependent way (Rana et al. 1990). Irradiation induces apoptotic cell death in NK cells (Seki et al. 1994).

Combined effects of radiation and immunological system on tumor cells: In recent years, cancer immunotherapy has seen some outstanding success, including immune checkpoint blockade therapies by monoclonal antibodies targeting inhibitory molecules on either immune effector cells or tumor cells (Hoos and Britten 2012, Pardoll 2012, Harris and Drake 2013, Yang 2015). Even if significant improvements of overall and progression-free survival are achieved in individual cancer patients, long-lasting tumor rejections in large patient groups are lacking. This might be due to high heterogeneity of different tumor types as well as poor immunogenicity of tumors and escape from immune recognition based on the concept of cancer immunoediting (Kwilas et al. 2012, Soukup and Wang 2015).

Radiation therapy has proved to be an intriguing partner for cancer immunotherapy in empowering the immune system (Kwilas et al. 2012).

Most importantly, radiation induces a clear increase in tumor associated antigen (TAA) quantity and quality via cancer cell death and enhanced protein translation (Soukup and Wang 2015). These changes, in turn, permit APCs, like DCs, to effectively activate T cells. In addition, damage-associated molecular pattern (DAMP), through immunogenic tumor cell death, activates DCs. DCs orchestrate a potent anti-tumor response (Roses et al. 2014). Persistent DC activation is critical in inducing a potent anti-tumour immune response, whereas immature DCs induce anergy or deletion of antigen-reactive T cell cytotoxicity. Radiation provides antigens, leading to the activation of signals, and it hinders the immunoediting process (Frey et al. 2014).

In addition, radiation recruits and enhances cytotoxic $CD8^+$ cells (Lim et al. 2014). Radiation also induces type I and II interferons and chemokines, like CXCL16, leading to enhanced intratumoral numbers and cytolytic activity of effector T cells (Matsumura et al. 1998, Kwilas et al. 2012,). Radiotherapy also affects normalization of tumor vasculature by increasing expression of chemokines CXCL9 and CXCL10 and vascular cell adhesion protein 1 (VCAM-1), leading to vessel remodeling and thus facilitation of T cell migration into tumors (Kershaw et al. 2013). Furthermore, radiotherapy increases tumor-specific antibody levels and induces production of pro-inflammatory cytokines, inducing activation and migration of various immune cells (Soukup and Wang 2015).

The possibilities for combining radiotherapy and immunotherapy are endless. Possible radiotherapy strategies include external and internal therapies, bone-seeking radionuclides, radiolabeled antibodies, and proton therapies (Soukup and Wang 2015, Bhattacharyya et al. 2016). They can be combined with immune checkpoint inhibitors, unspecific stimulation, vaccine-based combinations, adoptive effector cell transfer, or targeted immunotherapeutics, such as antibodies. The possibilities for variation include dosage, timing, and selection of patients bearing appropriate cancers showing a reasonable stage of disease.

Preclinical and clinical studies have been conducted using radiation combined with antibodies targeting immune blockade molecules like CTLA-4 and PD-L1 as well as IL-2 application (merged). Seung et al. treated metastatic melanoma and renal cell carcinoma patients with a combination of stereotactic body radiotherapy and high dose IL-2. Radiation consisted of one or two fractions of 20 Gy. They

observed a 67% response rate in patients treated with the combination therapy compared to historical 15% with IL-2 monotherapy (Seung et al. 2012).

Formenti and Demaria observed in 30% of patients an abscopal response in metastatic solid tumor patients treated with granulocyte macrophage–colony stimulating factor (GM-CSF) and local radiotherapy (Formenti and Demaria 2009, Formenti and Demaria 2013). The abscopal radiation response is characterized by the regression of the tumor at sites distant from the primary radiation fields (Kalbasi et al. 2013).

Abscopal effect is different from radiation-related bystander effect. Bystander effect is defined as the induction of biological effects in cells that are not directly traversed by a charged particle but are in close proximity to cells that are, or that have received signals from these irradiated cells (Fang et al. 2016).

However, it is known that radiation might have an immunosuppressive effect by proportionally increasing the inhibiting Treg cells. Studies indicate that regulatory T cells and induction of transforming growth factor β both inhibit radiosensitivity and immune-activating effects of radiation (Formenti and Demaria 2009, Formenti and Demaria 2013). Local high-dose radiation induces expression of co-inhibitory PD-L1. Theoretically, this is a reason to combine radiotherapy and immune checkpoint inhibitors (Kwilas et al. 2012).

Regarding the endless therapy possibilities, Soukup stated in 2015 that the devil seems to lie in the details of overcoming the tumor's ability to suppress and manipulate the immune system in order to maintain a state of immune evasion. The delicate balance between immunosuppression and immune activation determines whether a certain therapy will result in successful tumor elimination (Soukup and Wang 2015).

Combined effects of radiation and hyperthermia on tumor cells: The rationale of combining radiation and hyperthermia includes several factors. Due to more sluggish blood supply, some tumors may become hotter than normal tissues in the hyperthermia treatment field. Tumors may contain cells that are hypoxic, at low pH, and nutrient deficient. These conditions make them more radiation-resistant but susceptible to hyperthermia. Some tumor cells may be intrinsically more thermosensitive than normal cells. The S-phase in the cell cycle is particularly heat sensitive but resistant to radiation (Field 1987). Radiation resistance of tumor cells is related to the capacity of cells to repair sublethal DNA radiation damages. Hyperthermia prevents the proteins involved in DNA replication (Raaphorst 1992, Lauber et al. 2015). Thus, hyperthermia treatment after irradiation results in higher

inhibition of DNA repair than before radiation. Thermoradiosensitization could be achieved with low LET radiation but not with high LET radiation (Raaphorst 1992). Hyperthermia enhances the immunogenicity of irradiated tumor cells. However, very low doses of hyperthermia given at low temperatures immediately after irradiation can increase the rate of repair of DNA strand breaks (Dikomey 1982). Treatment of tumor cells with a combination of radiation and hyperthermia leads to enhanced tumor cell necrosis. The resulting cell debris and release of danger signals stimulate immune mechanisms. HSP70 expressed on tumor cells promotes tumor immunogenicity via DCs and releases pro-inflammatory cytokines (Lauber et al. 2015).

Kim et al. treated several tumor cell lines with heat shock and ionizing radiation. The combination increased the expression of NKG2D ligands on tumor cells; therefore, HSP70 was induced by heat shock but not by radiation. Elevated expression of NKG2D ligands render target cells sensitive to NK cell attacks (Kim et al. 2006).

Datta et al. cited 38 clinical trials with 1761 patients presenting cancers of breast, cervix, head and neck, rectum, urinary, bladder, esophagus, and different melanomas. The patients were treated using radiotherapy or thermoradiotherapy. An overall local complete response rate of 54.9% was achieved with thermoradiotherapy; with only radiotherapy the rate was 39.8% ($p < 0.001$). Adding hyperthermia to radiotherapy caused no significant increase in acute or late toxicity (Datta et al. 2015). In a prospective randomized, multicenter trial, van der Zee et al. treated patients presenting locally advanced pelvic tumors using radiation or radiation combined with hyperthermia. Cervical cancer patients treated with the combination demonstrated a complete response rate of 83% compared to 57% for the radiation alone group ($p = 0.003$). The figures for three years overall survival were 51% and 27%, respectively (van der Zee et al. 2000).

Local tumor hyperthermia as immunotherapy: Hyperthermia can increase the visibility of tumor cells to the immune system. The fever-range temperatures in vitro increase surface expression of MICA, an NKG2D ligand (Toraya-Brown and Fiering 2014) and surface MHC class I (Ostberg et al. 2007). Thus, tumor cells are better recognized and lysed by NK and $CD8^+$ T cells (Ito et al. 2003, Ostberg et al. 2007). Heat upregulates heat-shock proteins on tumor cells. Hsp70 has an epitope that stimulates NK cell proliferation and cytolytic activities. Furthermore, released Hsp70 binds directly with TLR2 and TLR4 on antigen-presenting cells like DCs. Hsp70 release starts at 41°C, reaches maximum at 43°C, and is diminished at 45°C

(Ito et al. 2005). However, some HSPs, like Hsp90, block apoptosis by directly interacting with and repressing the tumor-suppressing protein p53 (Lin et al. 2008). Thus, some HSPs activate the immune system to attack the heated tumor cells, while others prevent the heated cells from dying from the heat itself or from immune attack. Therefore, the overall outcome is influenced by the sum of all the effects of different HSPs (Toraya-Brown and Fiering 2014).

Exosomes, small membrane vesicles released by cells, act in intercellular communication. Tumor-cell-derived exosomes contain enriched amounts of tumor antigens, which are immunostimulatory via the DC system (Wolfers et al. 2001, Clayton and Tabi 2005). Exosomes from heated tumor cells containing a variety of chemokines stimulate anti-tumor immune responses by DC and CTC systems. Conversely, tumor-derived exosomes may be immunosuppressive, triggering apoptosis of T cells and inhibiting NK and CTC cells by blocking the NKG2D receptor (Clayton and Tabi 2005). Hyperthermia also has direct immunostimulatory effects on immune cells. At fever-range temperatures, NKG2D proteins are clustered on NK cells and IFNγ production of CTC is stimulated and DCs activated (Toraya-Brown and Fiering 2014).

In situ, local hyperthermia increases the permeability and diameter of tumor vasculature, resulting in better tumor transfusion and increasing pO_2. Also, immune cell trafficking is facilitated and intratumoral concentration of IL-6 increases. However, at temperatures higher than 43°C, the beneficial effects might be abrogated by hemorrhage. Local hyperthermia therapy amplifies effects of other immunotherapies like checkpoint blockade and adoptive T cell therapy (Toraya-Brown and Fiering 2014). Hyperthermia at 42°C–45°C and at ablation temperatures >45°C has been used mainly in in vitro and animal immunological studies. An increased intra-tumoral infiltration of NK cells, $CD8^+$ and $CD4^+$ cells, and DCs as well as induction of many cytokines and chemokines has been shown. In addition, the restrictive effect on distant metastases and resistance against tumor rechallenge is demonstrated (den Brok et al. 2004, Bear et al. 2013, Toraya-Brown and Fiering 2014).

3 Purpose of the study

The purpose of the study was to investigate effects of radiation, hypo-, and hyperthermia on the viability and cytotoxicity of natural killer cells and their subclasses. In addition, the ability of IFN α, β, γ, and IL-2 to prevent or restore damages made by radiation, hypo-, and hyperthermia and by their combinations was examined.

Specific aims were:

- To select the proper method to enrich non-selected NK cell populations and highly purified $CD56^+$ and $CD16^+$ subgroups assessed by yield, viability, purity, and cytotoxicity (Study I).

- To examine the radiation sensitivity of non-selected NK cell populations and $CD56^+$ and $CD16^+$ subgroups by single and fractionated irradiation using as endpoints viability and cytotoxicity by different methods. To describe effects of radiation using several mathematical models (Study II).

- To elucidate the prevention and restoration of radiation damages on viability and cytotoxicity on non-selected NK cells by IFN α, β, γ, and IL-2, and to illustrate the effects using mathematical models (Study III).

- To evaluate the thermal effects on cytotoxicity of non-selected NK cells in temperatures ranging from 31°C to 45°C and to examine the effects of IFN α, β, γ, and IL-2 in abolishing thermal damages. To further characterize the combined effect of irradiation and hyperthermia on NK cell cytotoxicity and to determine the ability of IL-2 to prevent and restore the damages (Study IV).

4 Materials and methods

4.1 Natural killer cells and permissions from the Finnish Red Cross Blood Service

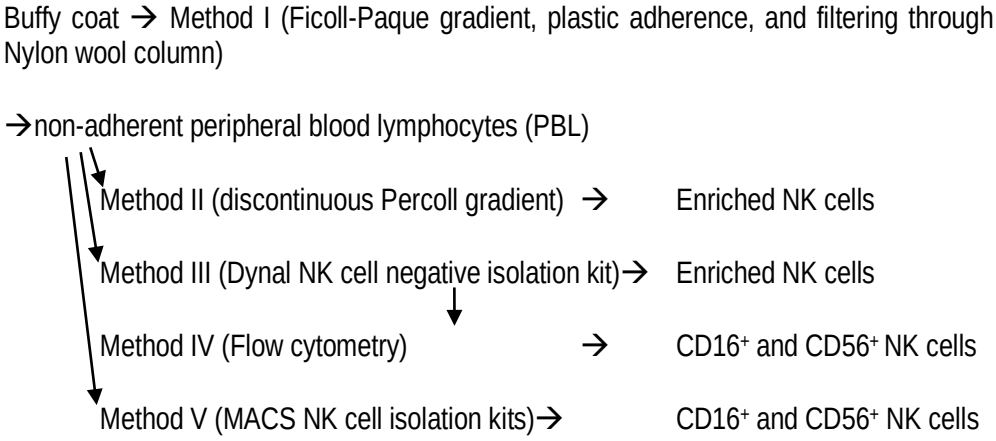
Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats remaining from the processing of blood collected from healthy blood donors. Buffy coats were obtained from the Finnish Red Cross. The ethical committee of the Finnish Red Cross Blood Service approved the use of these samples for this project in accordance with Finnish law (customer number 6129, approval number 331/2013). Before blood donation, the donors were informed that blood samples that were not required for patient treatment could be used anonymously for research, if permission from the Finnish Red Cross Blood Service was obtained.

4.1.1 Enrichment methods of natural killer cells and subpopulations

The starting materials for enriching NK cells from human peripheral blood mononuclear cells (PBMCs) were buffy coats (50 ml) obtained from the Finnish Red Cross transfusion laboratory, Tampere and Helsinki, Finland. Buffy coats were prepared on the day of use.

Three different methods were used to enrich total NK cells (methods I–III) and two methods to purify the $CD16^+$ and $CD56^+$ NK cell subsets (methods IV and V). The isolation scheme is shown in Figure 3.

Figure 3. Isolation schemes used to enrich human NK cells from buffy coats.



Adapted from study I of this dissertation (Hietanen et al. 2016, p. 72).

Method I. A method modified from Saksela et al. (1979) was applied to enrich NK cells from PBMCs. Briefly, the PBMCs were isolated using a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) as previously suggested by Böyum (Böyum 1968.). The resulting cells were incubated in 75 cm^2 Falcon flasks (BD Biologicals, MA, USA) for one hour. The cells that did not adhere to the plastic were filtered through a nylon wool column (Cellular Products, NY). The non-adherent peripheral blood lymphocytes (PBLs) were eluted in prewarmed medium and subjected to further enrichment steps and experiments (studies I–II).

Method II. A discontinuous Percoll density gradient was used as proposed by Timonen and Saksela (1980). Briefly, non-adherent PBLs from buffy coats were isolated as described for method I. The cells were subsequently centrifuged through a discontinuous Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient (steps of 2.5% from 50% [bottom] to 37.5% Percoll [top]) in 15 ml conical tubes (Sarstedt, Nümbrecht, Germany). The tubes were centrifuged at 300 g for 45 min at room temperature, and the fractions were collected from the top. Most of the NK cells were found in fractions 1 and 2 (studies I–IV).

Method III. Here also, non-adherent PBLs isolated by method I were used. The cells were further processed with a Dynal NK cell negative isolation kit, which magnetically depletes non-NK cells from the sample, according to the

manufacturer's instructions (Dynal Biotech ASA, Oslo, Norway) (Kai et al. 2004). The resulting NK cells were used in subsequent experiments (studies I–II).

Method IV. A flow cytometer (FACS Aria, BD Biosciences, CA, USA) was used to separate the $CD16^+$ and $CD56^+$ subsets from pre-enriched NK cells obtained using method III (Dynal isolation kit) to increase the purity of the NK cells. The antibodies used were an R-phycoerythrin (r-PE)-conjugated mouse anti-human $CD16^+$ monoclonal antibody and an allophycocyanin (APC)-conjugated mouse anti-human $CD56^+$ monoclonal antibody (BD Pharmingen, CA, USA) as described previously (Patrikoski et al. 2014) (studies I–II).

Method V. Non-adherent PBLs were isolated as described in method I. The $CD16^+$ and $CD65^+$ NK cell subsets were purified via magnetic depletion of non-NK cells and via positive selection using magnetic-activated cell sorting (MACS) $CD56^{dim}CD16^+$ and $CD56^{bright}CD16$- NK Cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (Deniz et al. 2002). In this study, $CD56^{dim}CD16^+$ and $CD56^{bright}CD16$- NK cells are referred to as $CD16^+$ and $CD56^+$ NK cells, respectively (study I, II).

4.2 Measurements of study endpoints

4.2.1 Cytotoxicity and viability assays

^{51}Cr release assay: The cytotoxicity of NK cells was measured using the modified ^{51}Cr release assay described by Timonen et al. (1981). Briefly, $Na_2{}^{51}CrO_4$-(PerkinElmer Health Sciences, Groningen, The Netherlands) labeled K-562 target cells (Lozzio and Lozzio 1975) were used at a target-effector ratio (T:E) of 1:12.5, which was selected after optimization in pilot studies. The experiments were performed in triplicate in a 96-well round-bottomed microtiter plate (Falcon BD, NJ, USA). The plates were incubated for 12–18 hours in a humidified atmosphere at 37°C. The incubation time was selected based on this study's preliminary experiments and reports of Saksela et al. (1979), Vanherbergen et al. (2013), and Zaretskaya et al. (1983). The supernatants were harvested and measured in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The relative cytotoxicity was calculated using the following formula: Cx (%) = ((exp.-spont.)/(max.-spont.) x 100)), where exp. = average of the experimental wells,

spont. = spontaneous release and max. = maximal release from K-562 cells lysed with 1% Triton X-100. The mean value of the triplicate samples was recorded. Spontaneous release was usually less than 10% of maximal release. The results were expressed as a percentage relative to the non-irradiated controls.

In some cases, a single radiation dose of approximately 40 Gy or higher resulted in the lysis of a portion of the NK cells. In these cases, less ^{51}Cr was released from the target K-562 cells than in spontaneous release, generating a negative result on the ^{51}Cr release assay. In such cases, the negative values were replaced with zeroes in further calculations (studies I–IV).

Trypan blue assay: To assess dead cells, a modified trypan blue exclusion assay described by Pappenheimer (1917) was used. To perform the post-irradiation assay, 0.1 ml of effector cells irradiated with different doses was added to wells of round-bottomed microtiter plates (NUNC, Roskilde, Denmark) in combination with 0.1 ml of target K-562 cells at an effector:target ratio of 12.5:1. The plates were incubated at 37°C in a humidified atmosphere for 12–18 hours. Next, 0.1 ml of 0.2% trypan blue was added, and the plates were incubated for 10 min. The blue (dead and damaged) effector NK cells and target K-562 cells were counted under a microscope. The results were expressed as a percentage of dead K-562 and NK cells of the controls (studies I, II).

Propidium iodide assay: Viability was also measured by flow cytometry using propidium iodide (PI) (Sigma Chemical Co., MO, USA) as described previously (Malygin et al. 1994). Briefly, isolated lymphocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies, and PI was added. The dead cells were measured using flow cytometry based on the red fluorescence, while viable lymphocytes exhibited green fluorescence. The results were expressed as a percentage of PI-stained cells, i.e., dead cells, relative to controls (studies I, II).

Adenosine triphosphate (ATP) assay: The viability of non-selected lymphocytes was measured with a bioluminescence method for intracellular ATP using commercial kits from LKB Wallac (Turku, Finland). Triplicates were used.NK cells were first disrupted with trichloracetic acid, then buffered with Tris-EDTA, and an ATP monitoring reagent (luciferin-luciferase suspension) was added. The amounts of ATP were then measured with the LKB WALLAC 1251 luminometer (LKB Wallac, Finland), and expressed as mV. Results were calculated as a percentage of values of corresponding non-irradiated control NK cells. ATP measurements were performed six hours after irradiation in experiments where NK cells were preincubated with IFNs or IL-2, and immediately after the

incubation time in experiments where the NKs were irradiated before incubation with these cytokines. In split-dose studies, ATP determinations were performed six hours after the last radiation dose. In recovery experiments, ATP levels were followed for up to five days (studies II, III).

4.3 Treatment of natural killer cells

4.3.1 Irradiation

In the early phase of radiation experiments, the enriched NK cell populations were gamma-irradiated in a ^{137}Cs device (Gammacell 2000, Mølsgaard, Denmark) at a dose rate of 4.1 Gy/min at room temperature (20°C). The cells were irradiated in 5 ml snap-cap U-bottom plastic tubes (BD Falcon, NJ, USA) containing 1.25×10^6 stirred and well-oxygenated NK cells in 1 ml of medium. The gamma-irradiation dose was controlled via lithium fluoride thermoluminescence dosimetry. In the later phase of radiation experiments, irradiation was performed using a clinical radiotherapy accelerator (Varian TrueBeam STx, Varian Medical Systems Inc., Palo Alto, CA) using a 6 MV photon beam and a dose rate of 6 Gy/min. The cells were irradiated at a uniform dose in 2 ml U-bottom Eppendorf ampoules (Sarstedt, Nümbrecht, Germany) containing 1 ml of medium immersed in the water phantom at 20°C.

For the dose calculation, the water phantom containing the plastic tubes was imaged using a computed tomography (CT) scanner (Toshiba Aquilion LB 1, Toshiba Medical System, Tokyo, Japan) at a slice thickness of 3 mm. The monitor units needed for the reference dose were calculated using an analytical anisotropic algorithm (AAA) (Eclipse, v. 10.0, Varian Medical Systems Inc., Palo Alto, CA, USA). The γ-rays from the ^{137}Cs device and the X-rays from the clinical radiotherapy accelerator have similar dose equivalent factors.

The daily single radiation doses used during radiation therapy typically range from 1.8–4 Gy; in stereotactic therapy the dose can be as high as 25 Gy. This dose range and non-therapeutic doses of up to 100 Gy were used to study the entire dose-response range of post-irradiation viability and cytotoxicity.

In the split-dose studies, doses of 10 and 30 Gy were selected from the upper and lower linear part of the semi-logarithmic section of the dose-response curve.

These doses were split into two fractions, applied at intervals from 0 to 24 hours or three equal fractions applied at three-hour intervals (studies II, III).

4.3.2 Thermal treatment

NK and target K-562 cells were suspended in 1 ml of medium and incubated at different temperatures in a water bath or an incubator in a 5% CO_2 and humified atmosphere for time periods typically ranging from 0 to 180 min. Preliminary studies showed no differences between the results obtained using a water bath or an incubator. Temperatures were measured using a Fluke 51 K/J thermometer (John Fluke, Everett, WA, USA) with an accuracy of +/- 0.1°C, and pH was controlled with phenyl red present in the medium. Cytotoxicity measurements were performed immediately after the thermal treatment, with the exception of the recovery studies, for which they were performed up to 72 hours after the thermal treatment (study IV).

4.3.3 Cytokine treatments

Recombinant IFN α2b (Schering-Plough, Kenilworth, NJ, USA), recombinant IFN β (Kyova Hakko Kogyo Co., Ltd., Tokyo, Japan), recombinant IFN γ (Genenteck, San Francisco, CA, USA), and recombinant IL-2 (Janssen Biochimica, Beerse, Belgium) were used. Recombinant IFN α2b, IFN β, IFN γ and IL-2 are referred to as IFNα, IFNβ, IFNγ and IL-2, respectively. In brief, samples of 1.25 x 10^6 NK cells/0.9 ml medium were incubated with 0.1 ml IFNs or IL-2 at various concentrations. Controls were treated in the same manner, with 0.1 ml of 0.9% NaCl instead of cytokines. NK cells were incubated with IFN α, β, and γ at concentrations ranging from 1 to 1000 IU/ml for 24–72 hours at 37°C before or after heat treatment. NK cells were incubated with IL-2 for times ranging from 0 to 140 hours at concentrations from 1.75 to 450 IU/ml before or after heating at 37°C and during heating at the indicated temperatures (studies III–IV).

4.4 Calculation of the radiobiological parameters

The following standard mathematical radiobiological models were used to quantify the radiation dose-response curves:

1) The linear-quadratic model $S = \exp(-(\alpha D+\beta D^2))$, where S represents the surviving fraction and D is the radiation dose in Gy. The linear-quadratic model has two inactivation parameters, the linear term α (Gy^{-1}) and the quadratic term β (Gy^{-2}) (Kellerer and Rossi 1971, Bentzen and Joiner 2009, Kellerer and Rossi 2012). When negative values were obtained for β, and thus biologically meaningless, the negative values were assigned a value of zero and the linear-quadratic model was refitted as suggested by Fertil et al. (1984).

2) The mean inactivation dose $\overline{D}$ (Gy) $= \int S(D)dD$ represents the area under the survival curve (AUC) in linear coordinate representation, where S(D) represents the survival probability and D the radiation dose. The AUC was not calculated from the fitted linear-quadratic model but by direct integration of the dose-response curve in question (Kellerer et al. 1976, ICRU 1979, Fertil et al. 1984, Fertil et al. 2012).

3) The single-hit multi-target model $S = 1-(1-\exp(-D/D_0)^n)$, where S is the surviving fraction, D is radiation dose, D_0 is the dose that decreases the number of viable cells to $\exp(-1)$ of its original value, and n is the extrapolation number.

The models were fitted to the experimental data using the OriginPro 2015 program (OriginPro Corporation, Northampton, MA, USA) (studies II, III).

4.5 Culture conditions

The cells were cultured and experiments (studies I–IV) were conducted at 37°C or at a selected temperature in a humidified air atmosphere containing 5% CO_2. RPM 1640 medium (Orion Diagnostica, Vantaa, Finland) was supplemented with 10% heat-inactivated fetal calf serum (FCS, Flow Laboratories, Irvine, Scotland, UK), L(+) glutamine (0.3 g/l) (Fluka, Buchs, Switzerland), and gentamycin (20 mg/ml) (Flow Laboratories, Irvine, Scotland, UK) and this combination is referred to as "medium" throughout these studies.

4.6 Statistical analysis

Data were analyzed with an SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA). Results were presented as mean ± SD. Statistical significance among the group means was assessed by one-way analysis of variance (ANOVA). Post hoc testing was performed with Bonferroni's modification of the t-test. The Kruskal-Wallis rank sum test was used as an alternative when the data were inappropriate for ANOVA analysis (e.g., data were not normally distributed). The constituent ratios were compared using the $\chi 2$ test (Chi-square test) and the Fisher exact test. Non-parametric correlations were calculated using Spearman's rho analysis. Differences were considered significant at $p < 0.05$.

5 Results

5.1 Study I: Post-irradiation viability and cytotoxicity of natural killer cells isolated from human peripheral blood using different methods

Enrichment of NK cell populations: The mean output from one buffy coat was $144.3 \pm 143 \times 10^6$ non-adherent PBL. The very high standard deviation reflects significant differences in the cell yields from buffy coats between donors. The yield of non-selected NK cells using method II was significantly higher than that using method III ($p = 0.012$). For highly purified NK cells, methods IV and V were equally effective ($CD56^+$ $p = 1.000$ and $CD16^+$ $p = 0.151$).

NK cell purity in enriched populations: The purity of the cell populations isolated using different methods was evaluated using flow cytometry following antihuman $CD16^+$ and $CD56^+$ antibody staining, whereas other NK cell subclasses were not assessed. Methods I–III equivalently enriched non-selected NK cells ($p = 0.323$). Methods IV and V isolated these cells more effectively ($p = 0.005$), yielding approximately 100% purity.

Yields from the enrichment methods: Method II produced significantly more $CD16^+$ cells than method III ($p = 0.033$). The yield of $CD56^+$ cells was equivalent between these two methods ($p = 1.000$). Methods IV and V were equally effective at harvesting $CD16^+$ ($p = 1.000$) and $CD56^+$ cells ($p = 1.000$). Relative to the total number of NK cells ($CD16^+$ and $CD56^+$) from 10^6 PBL from one buffy coat, there were significant differences ($p = 0.000$) among methods I–V. The yield was the highest for method V (method V vs. methods II, III, and IV: $p = 0.009$, 0.000, and 0.069, respectively).

Viability of NK cells isolated using methods I–V: The viability of the cell populations collected using methods I–V was assessed based on trypan blue exclusion. The viabilities of the cells isolated using methods I–III and V were identical ($p = 1.000$) and were greater than 95%, whereas the viability of the cells isolated using method IV was significantly lower than that of the cells isolated

using any other method ($p < 0.023$). These results indicated that flow cytometric cell sorting might reduce NK cell viability.

Cytotoxicity of isolated human NK cell populations: The cytotoxicity of enriched, non-irradiated NK cell populations was measured by the ^{51}Cr release assay. Two groups with different degrees of cytotoxicity were observed: (1) The non-selected NK cells enriched using methods I–III were equally cytotoxic ($p = 0.224$); and (2) the highly purified $CD16^+$ and $CD56^+$ NK cells by methods IV and V displayed equivalent killing capacity ($p = 0.471$). However, the NK cells isolated using methods IV and V were significantly less cytotoxic than those isolated using methods I–III ($p = 0.000$). The $CD16^+$ and $CD56^+$ cells isolated using methods IV and V exhibited identical levels of cytotoxicity ($p = 0.662$ and 1.000, respectively). Furthermore, there were no differences in cytotoxicity between the combined $CD16^+$ and $CD56^+$ cells and either NK cell subset alone ($p = 1.000$). Therefore, for the following experiments, the results from the $CD16^+$ isolated using methods IV and V as well as from $CD56^+$ NK cells were combined.

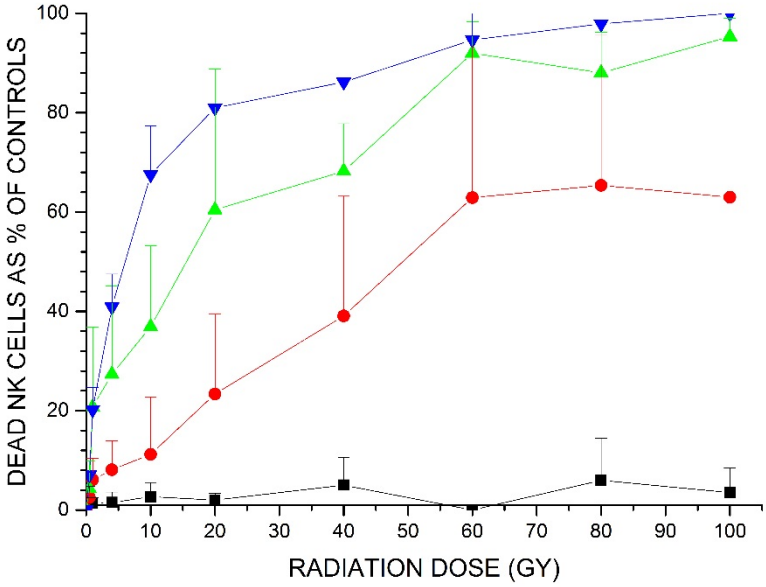
In addition, the cytotoxicity of non-irradiated purified NK cells was measured by counting dead K-562 target cells using the trypan blue method. No statistically significant differences were observed between the NK cell groups ($p = 0.820$). Thus, the trypan blue exclusion method applied to the target K562 cells was less sensitive than the ^{51}Cr release assay in detecting differences in NK cell cytotoxicity between the isolated populations.

Effects of irradiation on the survival of an enriched human NK cell population as a function of dose and time: The radiation sensitivity of non-selected NK cells was studied as a function of dose and time. The cell population enriched using method II was selected, because its cell output was higher and its purity was statistically comparable to that of the other non-selected NK cell preparations. For viability assessment, the trypan blue exclusion and propidium iodide methods were used as endpoints. The viability data measured using both methods at all single-dose radiation levels (from 0 to 100 Gy) were statistically similar ($p = 1.000$-0.100).

The death of NK cells was followed over a post-irradiation incubation period of 42 hours using the trypan blue exclusion method (Figure 4, p. 102). There were no recovery detectable. The survival decreased as a function of dose and time after irradiation. However, even at the highest dose of 100 Gy, at two hours post-irradiation, more than 95% of the NK cells were not stained blue; e.g., the NK cells remained alive. At 2, 6, 18, and 42 hours post-irradiation, the average

percentage of dead cells following irradiation at a dose of 1 Gy was 1.3%, 6.1%, 20.7%, and 20.2%, respectively, and that following irradiation at a dose of 10 Gy was 2.7%, 11.6%, 16.4%, and 67.5%, respectively. Even at high doses (80–100 Gy), a long observation time was required for all of the NK cells to stain blue. For the following experiments, an incubation period of 18 hours was selected, and a dose range from 0–40 Gy, representing the linear portion of the dose-response curve on a semi-logarithmic scale, was used.

Figure 4. Percentage of dead NK cells as a function of single radiation doses and time after radiation.



Non-selected NK cells were irradiated using single doses from 0 to 100 Gy. The viability was controlled by the trypan blue exclusion method 2 (▪), 6 (●), 18 (▲) and 42 (▼) hours after incubation.

(Adapted from Hietanen et al. 2016, p. 75).

Effects of irradiation on the killing capacity of enriched NK cell populations: The cell populations enriched using methods I–V were irradiated

with a single irradiation dose varying from 0–40 Gy. The cytotoxicities of the cell populations enriched using methods I–III representing non-selected NK cells were comparable ($p = 0.353$), and in this respect these cells were more radiation-resistant than the highly purified $CD16^+$ and $CD56^+$ NK cell subsets ($p = 0.000$). Irradiating NK cells enriched using methods I–V with different doses showed that the cytotoxicity of the NK cells differs significantly between methods I–III and IV–V at dose levels 10–30 Gy.

In addition, the proportion of dead NK cells was measured by the trypan blue exclusion. Interestingly, in the dose range of 1–10 Gy, the proportion of dead NK cells was approximately 20–30%, whereas these cells induced a level of ^{51}Cr release, which was occasionally higher than that for the non-irradiated controls.

The Spearman's correlations were calculated between radiation dose, ^{51}Cr release, dead NK cells, and dead K-562 cells. The best correlation was between radiation dose and dead NK cells, the correlation coefficient being 0.921 (two-tailed). The coefficients between other variables were all between approximately 0.6 and 0.7, and were positive or negative depending on the combination tested.

Radiation sensitivity of NK cell populations based on the trypan blue exclusion method: There were no statistically significant differences in radiosensitivity between NK cell populations enriched using methods I–V based on the trypan blue assay ($p = 0.230$). The results from the ^{51}Cr release assay were compared with those from the trypan blue exclusion assay in NK cells enriched using method II. Both analyses were performed at the same time-point after irradiation and ^{51}Cr release assay incubation. Dead target K-562 cells were counted using the trypan blue exclusion method. The results of the ^{51}Cr release assay and the proportion of dead effector NK cells counted using the trypan blue method equally effectively described damage to NK cells caused by irradiation ($p = 0.800$) in terms of cell death and the loss of cytotoxicity. The trypan blue exclusion method was not able to detect the elevation of NK cell activity induced by low radiation doses. The proportion of dead K-562 cells was lower than the proportion of ^{51}Cr released by these cells. K-562 cells release ^{51}Cr earlier than they absorb the trypan blue dye and are thus counted as dead.

5.2 Study II: Effects of single and fractionated irradiation on natural killer cell populations: Radiobiological characteristics of viability and cytotoxicity in vitro

Calculation of the radiobiological parameters for the cytotoxicity of irradiated NK cell populations: The cytotoxicity of irradiated NK cells, enriched using methods I–V as presented in Study I, was measured using ^{51}Cr release assay. For the calculation of radiobiological parameters, the irradiation dose range from 0 to 40 Gy was selected, including the linear part of the semi-logarithmic dose-response curve. The parameters of the linear-quadratic model (α and β) are presented in Table 3

Table 3. The α and β values of the linear-quadratic model for cytotoxicity data of irradiated NK cells enriched by different methods.

ENRICHMENT METHOD	α (Gy^{-1})	±s.e.	β (Gy^{-2})	±s.e.	p
METHOD I	0.03199	0.008	-2.57E-05	0.000	0.000
METHOD I *	0.0313	0.003	0	0	1
METHOD II	0.01362	0.030	0.00164	0.001	0.002
METHOD III	0.01532	0.001	0.00106	0.000	0.000
$CD16^+$ (COMB)	0.08985	0.029	-7.55E-04	0.001	0.009
$CD16^+$ (COMB) *	0.0732	0.011	0	0	1
$CD56^+$ (COMB)	0.0885	0.032	-3.66E-04	0.001	0.008
$CD56^+$ (COMB) *	0.0807	0.011	0	0	1

Linear-quadratic model parameters of the cytotoxicity data of the irradiated NK cells enriched by methods I-V. The $CD16^+$ data of methods IV and V were combined as well as $CD56^+$results. The cytotoxicity was measured using the ^{51}chromium release method The dose range was from 0 to 40 Gy. The cytotoxicity test was performed 18 hours post-irradiation. Standard errors of the parameters (± s.e.) and p-values are given.

* In the cases where β was negative and assigned to zero, a new α value was redetermined.

(Adapted from Hietanen et al. 2015, p. 1595)

It turned out that the β values were positive only under methods II and III, resulting in α/β ratios of 8.3 and 14.4 Gy^{-2} respectively. The values are typical for acutely responding tissues. The negative β values were replaced with 0 and the linear-quadratic model was recalculated. The difference between 0 and the negative β values (in the range of 10^{-4} to 10^{-5}) was not significant. The α values were from 0.01 to 0.03 for non-selected NK populations and from 0.07 to 0.08 for the combined $CD16^+$ and $CD56^+$ populations, respectively. The area under curve (AUC) values were calculated for the cytotoxicity data 18 hours after irradiation. The values for the non-selected NK cell populations were between 21 and 24 Gy and for highly purified $CD56^+$ and $CD16^+$populations approximately 13–14 Gy. However, the differences between populations were not significant ($p = 0.714$).

The trypan blue exclusion , propidium iodide and ATP methods were equally good in describing the viability of irradiated NK cells. The α and β values did not differ significantly.

Effects of fractionated irradiation were studied at different intervals and with different numbers of fractions on the cytotoxicity and viability of NK cells: The NK cells enriched using method II were irradiated with total doses of either 10 or 30 Gy. First, the total doses were split into two equal fractions applied at intervals of 1, 2, 3, 4, 8, 12, 16, 20, and 24 hours. At the 10 Gy level, cytotoxicity showed no significant changes between the fractions. At the 30 Gy level, a continuous but not significant rise was observed. The viability of NK cells measured using intracellular ATP levels showed no changes at the 10 Gy level. However, at the 30 Gy levels a significant rise of ATP content ($p = 0.002$) was observed between zero and six hours.

In the following experiment, the 10 and 30 Gy levels were divided into fractions of 2 x 5 Gy, 3 x 3.33 Gy, and 4 x 2.5 Gy at three-hour intervals. Correspondingly, 30 Gy was divided into 2 x 15 Gy, 3 x 10 Gy, and 4 x 7.5 Gy fractions. The cytotoxicity was significantly elevated ($p = 0.04$) when 10 Gy was divided into two fractions. Splitting 30 Gy into three fractions showed a 2.7-fold but not significant rise in cytotoxicity. The ATP levels were not affected significantly when 10 Gy was divided into fractions. However, splitting 30 Gy into the fractions indicated above showed a significant ($p = 0.001$) and continuous rise in the ATP levels.

5.3 Study III: Effect of cytokines IFN α, β, and γ as well as IL-2 on irradiated non-selected NK cell population

Natural killer cells were treated with recombinant IFNα, β, and γ and IL-2 using concentrations from 0 to 1000 U/ml before or after an irradiation dose of 30 Gy. Viability was measured using ATP as the endpoint and cytotoxicity using ^{51}Cr release assay. IFNα and γ used before irradiation decreased ATP levels 6%-16%; when used after irradiation, the decrease was about 60%. Thus, they sensitized NK cells to irradiation. There was no concentration-response relationship. In cytotoxicity studies, IFNγ demonstrated a concentration-response relationship and, at a concentration of 1000 U/ml, a significant elevation when used prior to irradiation. When used post-irradiation, IFNα, β, and γ showed no significant changes in cytotoxicity.

In contrast to IFNs, IL-2 demonstrated a clear concentration-response relationship in viability and cytotoxicity experiments. Pre-irradiation incubation with IL-2 showed a statistically significant elevation of ATP levels from the concentration of 100 U/ml ($p = 0.02$) upwards. Post-irradiation incubation produced no significant elevation of ATP levels.

In the cytotoxicity experiments, preincubation using IL-2 showed an even stronger concentration-response relationship. The ^{51}Cr release was elevated significantly from the concentration of 100 U/ml ($p=0.0007$) on. The concentration of 1000 U/ml increased the cytotoxicity 6.9-fold and as much as 1.13-fold over that of the non-irradiated control. The post-irradiation incubation using IL-2 in concentrations of 100 and 1000 U/ml elevated the ^{51}Cr release levels significantly ($p = 0.01$ and 0.01 respectively) when compared with irradiated controls. However, the levels were clearly lower than levels achieved using pre-irradiation incubation.

The possibility of protecting NK cells from irradiation was studied by splitting 30 Gy into two fractions of 15 + 15 Gy with four-hour intervals and pre-incubating the cells using various IL-2 concentrations. The concentrations from 10 to 1000 U/ml elevated viability 1.9 times and cytotoxicity 2.9 times. However, the elevations were not statistically significant. Thus, in this setting, combining split-course irradiation and IL-2 gave no additional protection against radiation damage in NK cells.

In addition, the study explored whether the combinations of IFNα, β, and γ with IL-2 would protect NK cell viability and cytotoxicity more than IL-2 alone.

NK cells were pre-incubated with 100 U/ml of IFNα, β and γ and IL-2 and irradiated using doses from 0 to 40 Gy. In both viability and cytotoxicity studies, no combination gave significantly better results than IL-2 used alone.

The possibility of assisting NK cell recovery from radiation damage was studied. The NK cells were incubated pre-and post-irradiation using IFN α, β, and γ as well as IL-2 in concentrations from 0 to 1000 U/ml. The viability by ATP and cytotoxicity by ^{51}CR release assay were followed for five days. There was no recovery in the intracellular ATP content or cytotoxicity. On the contrary, the parameters decreased as a function of time. However, high concentrations of IL-2 were able to slow down the decline of both parameters.

Radiobiological characteristics of IFN α, β, and γ as well as IL-2 treated and irradiated NK cells were determined. The NK cells were pre-incubated with 100 U/ml of IFN α, β, and γ as well as IL-2 for 72 hours and subsequently irradiated with doses from 0 to 40 Gy. The irradiation dose-response curves were calculated and the radiobiological characteristics of the single-hit multi-target, linear-quadratic, and mean inactivation dose models determined. The results are presented in Table 4

Table 4. Radiobiological characteristics of ATP (4 A) and ^{51}Cr-release (4 B) of irradiated, non-selected NK cells treated with IFN α, β γ and IL-2 (± SD).

4A.

ATP	D_0 (Gy)	± SD	n	± SD	α (Gy^{-1})	± SD	β (Gy^{-2})	± SD	$\overline{D}$	± SD
Control	20.3	4.5	2.0	0.4	0.020	0.006	0.0004	0.0003	30.6	9.3
IFNα	20.5	5.7	2.2	0.3	0.020	0.009	0.0005	0.0001	26.6	5.2
IFN β	16.9	1.9	1.7	0.1	0.010	0.007	0.0002	0.0005	23.1	1.8
IFN γ	27.9	15.9	1.6	0.7	0.010	0.010	0.0004	0.0005	32.3	12.9
IL-2	18.1	3.1	3.7	2.9	0.003	0.002	0.0005	0.0006	39.5	8.8

4B.

^{51}CR-release	D_0 (Gy)	± SD	n	± SD	α (Gy^{-1})	± SD	β (Gy^{-2})	± SD	$\overline{D}$	± SD
Control	25.8	11.8	1.7	0.3	0.010	0.0004	0.0002	0.0001	31.8	14.3
IFNα	19.8	9.4	2.1	0.6	0.030	0.0100	0.0002	0.0003	32.2	13.9
IFN β	13.9	6.4	3.3	1.7	0.020	0.0020	0.0009	0.0001	27.2	15.9
IFN γ	24.1	11.2	1.7	0.7	0.030	0.0300	0.0003	0.0001	31.7	17.2
IL-2	24.3	11.6	4.0	0.7	0.009	0.0003	0.0002	0.0003	51.4	13.3

Non-selected NK cells were incubated with recombinant IFNα, β, and γ and IL-2 (100 IU/ml) for 72 hours. Thereafter, the cells were irradiated with doses ranging from 0 to 40 Gy. The viability was evaluated measuring the intracellular ATP content and cytotoxicity by ^{51}Cr release assay. Dose-response curves were used to calculate the parameters D_0 and n of the single-hit, multi-target model, α and β of the linear-quadratic model and $\overline{D}$ (AUC) of the mean inactivation dose.

(Adapted from Hietanen et al. 1995, p. 123)

The radioprotective effect of IL-2 was expressed in the broadened "shoulder" and the elevated level of the radiation-dose-response plot in the viability and cytotoxicity experiments. In the ATP studies, α/β values for IFNα, β and γ treated NK cells were essentially in the same range than the control. IL-2 clearly lowered the α value so that α/β value, 6 Gy^{-1}, became typical of late reacting tissues. No such effect was seen in the ^{51}Cr release assay.

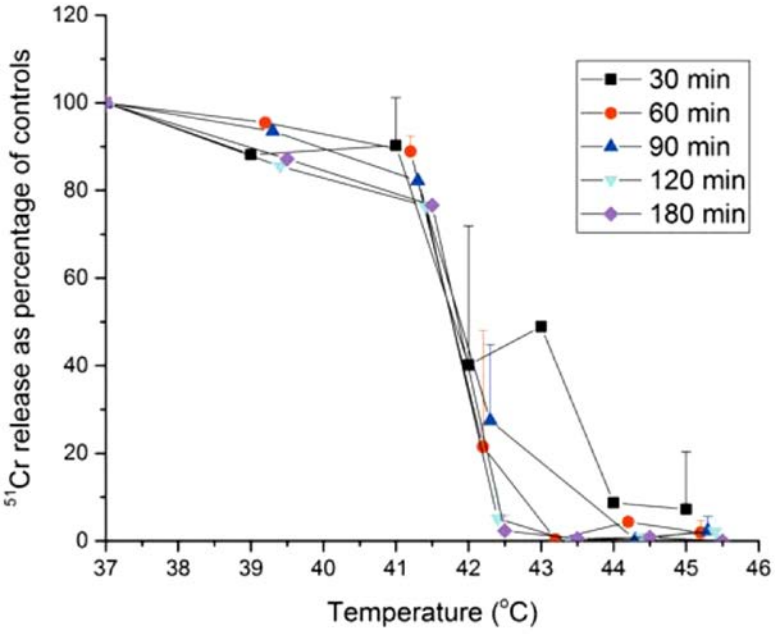
$\overline{D}$s for IL-2 treated NK cells were significantly higher ($p \leq 0.05$) than those for the controls, or with IFN α or β, but not with IFNγ-incubated cells. Also, in the cytotoxicity studies, $\overline{D}$s for IL-2 treated NK were higher than $\overline{D}s$ for all IFN treated cells ($p \leq 0.05$). The n in the curve presenting the ^{51}Cr release but not in the ATP data for IL-2 treatments was significantly higher than levels for control or IFN-treated cells.

5.4 Study IV: Restoring natural killer cell cytotoxicity after hyperthermia alone or combined with radiotherapy

Non-selected natural killer cells enriched using method II were subjected to temperatures from 31°C to 45°C for 180 min, and the cytotoxicity was examined using ^{51}Cr release assay. The cytotoxicity remained unchanged between 31°C and 37°C, followed by slow and continuous decrease. The most rapid reduction was observed between 41°C and 42°C. At 43°C, cytotoxicity was near zero.

In the following, NK cells were heated for periods from 30 to 180 min at temperatures from 39°C to 45°C. Temperature elevation from 39°C to 41°C did not significantly affect cytotoxicity at any heating times. However, an increase from 41°C to 42°C resulted in a clear reduction of cytotoxicity at all heating times. In the temperature range from 43°C to 45°C for heating times from 30 to 180 min the cytotoxicity was near zero (Figure 5).

Figure 5. The effects of different incubation times and temperatures on NK cell cytotoxicity



NK cells were incubated for 30, 60, 90, 120, and 180 min at temperatures ranging from 39 to 45˚C, and cytotoxicity was measured by ^{51}Cr release. The error bars represent the standard deviation of the experiments (n = 2-27)

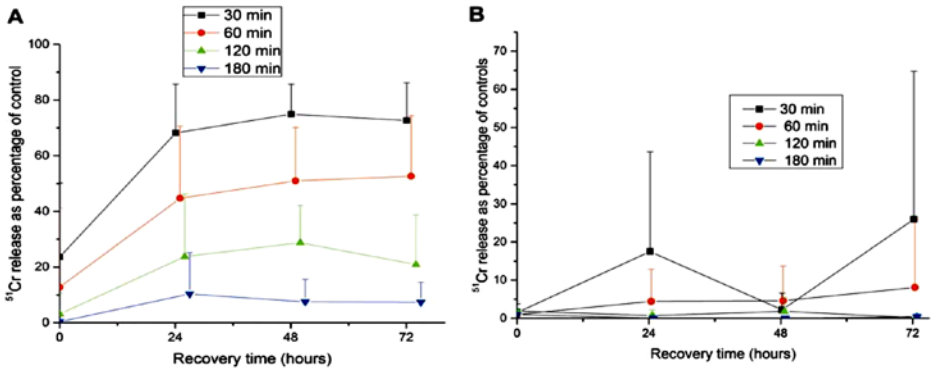
(Adapted from Hietanen et al. 2016, p. 558)

In addition, NK cells were incubated at 42°C for times ranging from zero to 180 min and their cytotoxicity measured. The cytotoxicity decreased almost linearly on a logarithmic scale, reaching almost 0% of non-heated control after 180 min.

The recovery of NK cell cytotoxicity was studied by heating NK cells at 42°C for times ranging from zero to 180 min. Thereafter, the cells were incubated at 37°C for 72 hours. For all heating times, the cytotoxicity recovered significantly at 24, 48, and 72 hours of recovery ($p = 0.01$, 0.01, and 0.01). The cytotoxicity elevated most remarkably during the first 24 hours, followed by a plateau. The degree of recovery was dependent on heating time. Seventy percent of cytotoxicity was restored at 72 hours for a heating time of 30 min (Figure 6A). The experiment

was also conducted at 45°C. After 72 hours, only 25% of controls had recovered after 30 min heating (Figure 6B).

Figure 6. Recovery of NK cell cytotoxicity after thermal treatment for different times at 42˚C (A) and 45˚C (B)



NK cells were incubated at 42˚C (A) and 45˚C (B) for 0, 30, 60, 120, and 180 min. Recovery times were 24, 48, and 72 hours at 37˚C. Cytotoxicity was measured. The error bars represent the standard deviation of the experiments (n = 4).

(Adapted from Hietanen et al. 2016, p. 558)

The role of IFN α, β, and γ in preventing and restoring thermal injury of cytotoxicity of NK cells was explored. The NK cells were pre-incubated with IFN α, β, and γ in concentrations from 1 to 1000 U/ml for 24 hours. Then, the cells were heated at 42°C for 180 min. The IFNs exhibited no statistically significant concentration-dependent effect on cytotoxicity. The same was observed when the NK cells were first heated and then incubated with IFNs.

To study the role of IL-2 in preventing and restoring NK cell cytotoxicity, the cells were pre-incubated with IL-2 concentrations from 0 to 450 U/ml for zero to 140 hours and then heated for 180 min at 42°C. IL-2 concentrations ≥56 U/ml markedly elevated the cytotoxicity over 100% of controls, reaching 1585% of controls at 450 U/ml of IL-2 at incubation time of 140 hours.

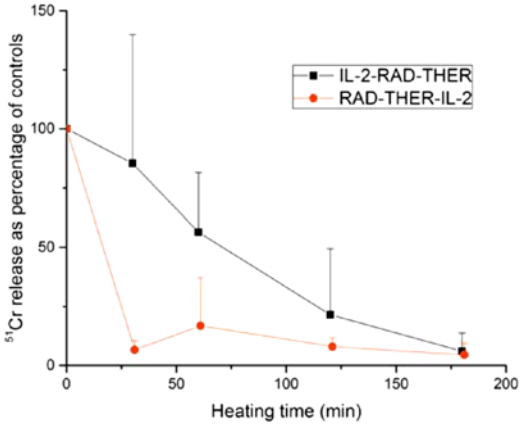
Next, the NK cells were incubated with IL-2 using a concentration of 450 U/ml for 18 hours before, during, and after heating at 41°C for 180 min. IL-2 elevated

the cytotoxicity levels in every case over the controls, and no statistically significant differences were observed between the groups. According to the results, interferons showed a very mild or no concentration-dependent effect on NK cell cytotoxicity, whereas IL-2 was clearly effective in a concentration and incubation time-dependent manner. Therefore, IL-2 was the only cytokine selected for the next experiments.

The following experiment examined the combined effect of hyperthermia and irradiation on NK cell cytotoxicity. The NK cells were irradiated with 20 Gy and then heated at 42°C for 30 to 180 min or vice versa. No statistically significant differences were found in NK cell cytotoxicity between the two approaches ($p = 0.520$). Next, the same approaches were used but with intervals of 0 to 180 min at 37°C between heating and irradiation treatments. In these experiments, no statistical differences were found in cytotoxicity at any interval times examined.

Earlier, IL-2 was shown to restore both irradiation and thermal injuries to NK cells. Therefore, the possibility of reversing the damages due to combined irradiation and thermal treatment on the cytotoxicity of NK cells was explored. NK cells were first incubated for five days with 100 U/ml IL-2 and then irradiated with 20 Gy and heated at 42°C for 0 to 180 min. Second, NK cells were incubated with IL-2 after irradiation and thermal treatment. A statistically significant difference in recovery of cytotoxicity was observed when IL-2 was applied before irradiation and heating ($p = 0.000$). The cytotoxicity was significantly improved with heating times of 30 and 60 min ($p = 0.030$ and 0.000 respectively) (Figure 7).

Figure 7. Recovery of NK cell cytotoxicity treated with IL-2 from combined irradiation and hyperthermia



The cytotoxicity of NK cells treated at 42°C using combinations of IL-2 treatment, irradiation, and hyperthermia. NK cells were incubated with 100 U/l IL-2 for five days at 37°C. The irradiation dose was 20 Gy. Heating times at 42°C were 0, 30, 60, 120, and 180 min. The following combinations were used: 1) IL-2, irradiation, and hyperthermia (IL-2-RAD-THER) and 2) irradiation, hyperthermia, and IL-2 (RAD-THER-IL-2). The error bars represent the standard deviation of the experiments (n = 3-12).

(Adapted from Hietanen et al. 2016, p. 560)

5.5 Summary of results

The key findings in studies I, II, III, and IV are presented in Table 5.

Table 5. Key findings of studies I-IV

Study I	NK cell enrichment method important Enrichment purity depends on method NK cell viability depends on enrichment method Cytotoxicity depends on enrichment method Low to medium size irradiation doses activate NK cell cytotoxicity
Study II	Radiobiological models used: linear-quadratic, single-hit multi-target, and mean inactivation dose models NK cells are acute reacting tissue type with α dominance Fractionated irradiation with varying doses and intervals showed inconsistent results
Study III	IL-2 abolishes single and fractionated radiation-induced damages on viability and cytotoxicity of NK cells in a dose-dependent manner IFN α, β, and γ demonstrate no dose-dependent effect on cytotoxicity and mostly insignificant changes
Study IV	Hypothermia produces no significant effect on cytotoxicity of NK cells Hyperthermia decreases NK cell cytotoxicity depending on temperature and heating time Most dramatic decrease of cytotoxicity occurs between 41°C and 42°C Most of the recovery of cytotoxicity takes place within 24 hours IL-2, but not IFNα, β, and γ, showed a concentration-dependent effect on preventing and recovering the cytotoxicity from hyperthermia The combination of hyperthermia and irradiation or vice versa showed no statistical differences in NK cell .cytotoxicity IL-2 was able to recover the irradiation and heating induced damages in cytotoxicity The effect was significantly better when IL-2 was applied before irradiation and heating

6 Discussion

6.1 Study I

Study I demonstrated that the NK cell enrichment methods I–IV clearly influenced the yield, viability, and cytotoxicity of resulting NK cells. It was not possible to find in the literature similar comparative studies of NK cell purification methods. The cytotoxicity of non-selected NK cells (methods I–III) was significantly higher than that of the highly purified $CD56^+$ and $CD16^+$ cells (methods IV–V). It is possible that the $CD56^+$ and $CD16^+$ cells cooperate, augmenting the cytotoxicity via cytokines and chemokines secreted by $CD56^+$ cells (Papamichail et al. 2004, Bryceson et al. 2006, Caligiuri 2008). The other possibility is that some other immunologically active cells are involved. In a preliminary study, only macrophages and B cells were found, representing 1% of all cells. Thus, this possibility is less plausible. It is more likely that purification methods used, especially flow cytometry, significantly decreased the viability and cytotoxicity of NK cells.

All the studies here are conducted in vitro using buffy coats of healthy persons. Large interindividual differences were seen in this and other studies (McGinnes et al. 1987, Louagie et al. 1999). Also in vivo, for example in cancer patients, these properties of NK cells might be different from those in these and other in vitro studies. In an individual patient, the balance between inhibiting and activating receptor signals may vary, as may ligand shedding (Marcus et al. 2014). Further, in the tumor cells, immunogenicity and the tumor microenvironment are different (Melvold and Sticca 2007). Thus, in vivo the NK cell activity may be increased or decreased down to anergy (Caligiuri 2008).

In earlier reports on in vitro NK cell irradiation experiments, the dose ranges have been from some cGy to 30 Gy (Zarcone et al. 1989). In this study, the dose range of irradiation was very large in order to cover reliably all doses used in daily radiation therapy. In addition, the non-therapeutic range up to 100 Cy was explored in order to detect possible phenomena caused by non-living cells or other factors. In the cytotoxicity and viability tests using ^{51}Cr release assay and trypan blue exclusion, respectively, most of the NK cell cytotoxicity was abolished by 30-

40 Gy. However, some cytotoxicity (figures 1A–1C, p. 40-41) and viability (figure 4, p. 102)was observed even at 100 Gy, when most of the NK cells were destroyed. Millard et al. showed that cytotoxic granules released from dead NK cells were cytotoxic to the target cells (Millard et al. 1984).

NK cells exposed to low radiation doses exhibit cytotoxicity higher than the non-irradiated controls. This is supposed to be a common adaptive reaction of cells irradiated with low doses. This phenomenon is extensively studied and it has been connected with radiation-induced hormesis, radioresistance, adaptive responses, and bystanding effect. Upregulation of the DNA repair mechanism, role of mitochondrial ATP production, prostaglandins and stress proteins are discused (Makinodan and James 1990, Rana et al. 1990, Rodel et al. 2012, Tang and Loke 2015).

Zaretskaya et al. studied NK cell activity of healthy male donors over five consecutive days. They observed considerable intra-personal daily variations. They concluded that one reason for this phenomenon was the ^{51}Cr release assays' duration. Sixteen hours, and not the four-to-eight-hour incubation period, was the optimal time to reduce this kind of variation (Zaretskaya et al. 1983). Similar incubation times are reported by others (Saksela et al. 1979, Vanherberghen et al. 2013). In this study series, incubation times from 12 to 18 hours were used.

6.2 Study II

In Study II, mathematical models were calculated to characterize the viability and cytotoxicity of irradiated NK cells enriched by methods I–IV. The viability was evaluated using trypan blue exclusion, propidium iodide, and ATP methods. These methods described different aspects of cell death. They were equally good in expressing cell death of irradiated NK cells.

The mathematical models to describe irradiation effects on the viability and cytotoxicity of different NK cell populations were the single-hit multi-target, the linear-quadratic (LQ), and the mean inactivation dose (the area under the curve, AUC) models. The models are based on stochastic mathematical models, even if the low irradiation region of the cell survival curve is stochastic but the higher radiation dose region is non-stochastic.

The LQ model is the most popular in daily cancer treatment when comparing the biological effects of radiation on cells (Bentzen and Joiner 2009). However, it is

not capable of meaningfully describing the increased cytotoxicity over non-irradiated controls at low irradiation doses. Further, there is still debate about whether the LQ model is relevant in the high-dose region as in this study. The discussion is ongoing (Santacroce et al. 2013, Kondziolka et al. 2015).

The α component describes best the initial linear part of the cell survival curve, reflecting the immediate, irreparable double-strand DNA damage by single-hit single-target effects (Gillespie et al. 1975, Chapman 2003). Theoretically, the α-type damages of irradiation would not allow recovery. In this paper, no recovery in viability was observed. The β component influences the survival curve at higher radiation doses, reflecting single-hit two-target killing.

In this study, viability and cytotoxicity of the irradiated NK cells showed α type responses typical of acutely responding cells and indicating irreparable damages. The β values were inconsistent. In the cytotoxicity studies, positive β values demonstrated only NK cells enriched by methods II and III, all others being negative indicating single-hit single-target killing. The viability data, measured by ATP, resulted in positive α and β values in the LQ model, indicating both single-hit single-target and single-hit two-hit killing. In all other methods, β was negative, showing a single-hit single-target mechanism. Negative β values were replaced by 0 and the linear-quadratic model was recalculated. However, the new α values were in the same range as the non-corrected ones. The α/β relations were characteristic for acutely responding tissues like lymphocytes (Brovall and Schacter 1981, Louagie et al. 1999, Heylmann et al. 2014). In cytotoxicity studies, the AUC values for methods I–III were higher than those for methods IV and V.

In fractionated irradiation, α component reflects best the initial linear part of the cell survival curve and the total dose, and not the dose per fraction. β mechanism again reflects the survival curve at higher radiation doses and the dose per fraction of radiation (Chapman 2003, Niranjan and Flickinger 2008). In this study, results of fractionated irradiation on viability and cytotoxicity were not unequivocal. When a total dose of 10 Gy was divided either in two fractions at different intervals or in several fractions, the viability measured using ATP was not significantly elevated. However, dividing a total dose of 30 Gy resulted in a significant rise of ATP levels using both ways of fractionation. The cytotoxicity was elevated only when the total dose of 10 Gy was divided in two fractions.

The single-hit multi-target model was quite able to best describe the shoulder region at the low-dose region of the dose-effect curve. The mean inactivation dose expressed as the area under the curve (AUC) included the elevations of viability

and cytotoxicity at low irradiation doses as well as at high-dose regions of the dose-response curve. In the single-hit multi-target model, D_0 and n are influenced by the high-dose region of the dose-response curve. The viability of irradiated NK cells was equally well described by AUC using ATP, PI, and trypan blue methods.

Only some in vitro studies have been published concerning radiobiological parameters of other lymphocytes. D_0 values are calculated for B (Thomson et al. 1994) and T cells (Williams et al. 1994).

The conventional daily irradiation single dose is approximately 2 Gy; the hypofractionated dose about 3 Gy; the hyperfractionated therapy dose between 1 and 2 Gy; and in stereotactic treatments the dose can be as much as 20 Gy. Thus, the treatment time for a typical clinical radiotherapy accelerator with a dose rate of 6 Gy/min is from 10 seconds to 200 seconds. Theoretically, a single NK cell in the blood stream passes the same treatment location once a minute for a radiation volume dependent time. Thus, the daily dose per one NK cell would be very small and would activate NK cells.

However, the in vivo results have been conflicting. Louagie et al. (1999) followed the number of NK cells before and after radiotherapy of 46–50 Gy in two Gy daily fractions. NK cells demonstrated a limited decline during the first weeks of therapy (Louagie et al. 1999). Steward and Ales found that some irradiated patients demonstrated elevated NK cell activity, whereas others showed subnormal levels. The activity was connected with the clinical course of the disease (Stewart and Ades 1984). Another group did not find any significant effects of radiotherapy on the NK cell activity (Kadish and Ghossein 1983). Blomgren again demonstrated decreased NK activity (Blomgren et al. 1982). McGinnes concluded that the effect of radiotherapy on the NK activity in cancer patients is dependent on the site of irradiation and not on the dose of radiotherapy or stage of the disease (McGinnes K. et al. 1987).

6.3 Study III

The radioprotective and recovery capability of IFN α, β, and γ as well as IL-2 on irradiated NK cells was studied. IFNγ had some degree of dose-dependent protection of the NK cell cytotoxicity. IFNα and β demonstrated no dose-dependent enhancement effect on viability, measured using ATP, and cytotoxicity, measured using ^{51}Cr release assays. On the other hand, pre-incubation with

recombinant IFN α, β, and γ decreased ATP levels of irradiated NK cells insignificantly. However, the levels of viability and cytotoxicity were slightly higher when IFNs were used before irradiation.

In contrast to IFNs, IL-2 showed a significant dose-dependent effect on both parameters studied when used either before or after irradiation. The different combinations of IL-2 and IFNs were not more effective than IL-2 alone in this respect. On the other hand, Franzese et al. showed that the combination of IFNβ and IL-2 successfully antagonized radiation damages in NK cells, mainly when IFNβ was used before IL-2. They irradiated whole peripheral blood with 20 Gy in vitro and thereafter isolated the mononuclear cells to incubate the cells with IL-2 and IFN α, β, and γ. Thereafter the cytotoxicity assays was performed (Franzese et al. 2013). The time intervals between cell irradiation and cytotoxicity tests, IFNs, and IL-2 concentrations and treatment schedules were different from this study. In addition, whole blood includes a variety of immunologically active cells and biologically effective molecules. In this study, purified, enriched NK cells were used.

IL-2 broadened the shoulder region of the dose-response curve describing the cytotoxicity of irradiated NK cells. The viability measured by ATP demonstrated α/β values typical of late reacting tissues. The shoulder region is reflected by the single-hit, multi-target model by increasing the n and D_q values as well as by the AUC values. The viability or cytotoxicity of irradiated NK cells declined as a function of time. IFNs used before or after irradiation were not able to recover viability or cytotoxicity. On the contrary, IL-2 was able to recover or slow down the decrease of these parameters.

In this in vitro study, recombinant IFN α, β, γ, and IL-2 were used in concentrations from 0 to 1000 IU/ml. IL-2 showed clear protective effect on NK cell viability and cytotoxicity in concentrations about 50–100 IU or more. IFNγ demonstrated some activity at 1000 IU/ml. Healthy persons demonstrate in vivo IL-2 levels from 8.9 to 15.5 pg/ml, depending on donor's age and anticoagulants used (Krishnan et al. 2014). According to the Chiron conversion formula for aldesleukin (a form of recombinant IL-2, approved in the US), 1.1 mg = 18 million IU (MIU). Thus, 1 IU = 61.1 pg/ml. Roughly, IL-2 levels in vivo would be much lower than those needed in vitro for significant changes in viability and cytotoxicity of NK cells.

Vuoristo et al. treated patients with melanoma and renal cell carcinoma using IL-2 2.4-4.8 x 10^6 2-3 times daily 5 times a week for 6 weeks and IFNα-2b. They

observed a positive connection between the median level of IL-2 and the median survival. The IL-2 levels were up to 1130 pg/ml (Vuoristo et al. 1996). This level is still too low for enhancing NK cell viability and cytotoxicity in vitro. In pharmacokinetic studies using human recombinant IL-2 10^6 IU/m^2, Konrad et al. typically observed 650 IU/ml, which is well able to protect and recover NK cells from radiation-induced injuries (Konrad et al. 1990).

Reports could not be found in the literature concerning effects of IFNs and IL-2 on irradiated T cells, B cells, macrophages, or other lymphocytes in vitro or in vivo expressed in radiobiological formulas.

In vivo, the cooperation of cytokines, chemokines, and angiogenic and growth factors is extremely complex. Moreover, this complexity involves immunological cells, tumor cells, various ligands, the vasculature and tumor microenvironment. Changing one component might cause multiple changes in this complex. Yurkovetsky et al. studied the effect of IFNα2b on 29 cytokines, chemokines, and angiogenic and growth factors in melanoma stage IIB–III patients. IFNα2b significantly decreased serum levels of immunosuppressive and tumor angiogenic/growth stimulatory factors (VEGF, epidermal growth factor, and hepatocyte growth factor) and increased levels of antiangiogenic IFN-gamma inducible protein 10 (IP-10) and IFNα (Yurkovetsky et al. 2007). Mission et al. studied 10 cytokines in patients with cervical intraepithelial neoplasia grades II–III treated with intralesional IFNα2b. In the responding patients, a great oscillation of cytokines was observed. The levels of IL-2 and IL-12 increased and IFNγ and TNFα decreased (Misson et al. 2011). It can be concluded that predicting the effect of one factor in vivo based on the results obtained in vitro might be difficult.

6.4 Study IV

NK cells demonstrated in vitro no significant changes in cytotoxicity at hypothermic temperatures between 31 and 37°C. However, in vivo, even a modest -0.45°C decrease in body temperature increased NK activity (Lackovic et al. 1988). Healthy men exposed to cold demonstrated a significant elevation of NK activity (Brenner et al. 1999). The difference in vitro and in vivo might be caused by the modulation of cytokine production in vivo (Rhind et al. 2001). Hypothermia upregulates the expression of IFNγ and IL-12 by modification of the pattern of cytokine gene expression (Arai et al. 2008). In turn, Castellani observed

downregulation of IL-2, IL-12, and IFNγ levels in cold exposure by an imbalance of Th1 and Th2 exposure (Castellani et al. 2002).

In this in vitro study, the cytotoxicity of NK cells decreased insignificantly between 37°C and 41°C. Several other investigators have reported similar effects on NK cell cytotoxicity (Azocar et al. 1982, Nurmi et al. 1982, Dinarello et al. 1986, Onsrud 1988). However, Ostberg et al. and Kubes et al. reported enhanced NK cell cytotoxicity at fever-range temperatures (Ostberg et al. 2007, Kubes et al. 2008). The heating times and different target cells may explain some of the discrepancies (Dayanc et al. 2008).

In this study, increasing the temperature from 41°C to 42°C demonstrated a heating time–dependent (from 30 to 180 min) dramatic decrease of NK cell cytotoxicity. The same phenomenon is seen also by others (Azocar et al. 1982, Nurmi et al. 1982, Dinarello et al. 1986, Onsrud 1988) and summarized by Dayanc et al. (Dayanc et al. 2008). At 42°C, heating times from zero to 180 min decreased the cytotoxicity linearly on a semi-logarithmic scale. At 180 min, hardly any cytotoxicity was detectable. The recovery from this thermal treatment occurred mostly within the first 24 hours. The degree of recovery was dependent on the heating time. Lipid rafts are important in the formation of NK cell synapses. Disruption of these rafts causes loss of the thermally enhanced NK cell cytotoxicity (Ostberg et al. 2007). In this study, NK cells heated to 42°C for different times showed a capability of recovering the cytotoxicity in a heating time–dependent manner. The findings confirmed results by Yang et al. (1991).

In in vivo studies, fever-range thermal stress enhances human NK cell cytotoxicity (Ostberg et al. 2007); thereby, both NK cells and tumor cells are affected at the same time. This enhancement is connected with NKG2D receptors on NK cells and plasma membrane reorganization (Dayanc et al. 2008). Tumor cells exhibit heat shock–induced proteins (HSPs). NK cells kill tumor cells bearing heat shock proteins. However, some HSPs prevent the tumor cells from dying from heat itself or from immune attack, as discussed by Toray-Brown and Fiering (2014). In addition, in vivo elevated temperatures positively affect blood flow and the tumor microenvironment. Hyperthermia may act as an adjuvant to the immune response (Dayanc et al. 2008).

In this in vitro study, IFNα, β, and γ demonstrated no dose-dependent activity in preventing or recovering thermal injuries in NK cell cytotoxicity. Onsrud reported similar results using leukocyte interferon (IFNα) (Onsrud 1983). In vivo, Robins et al. treated cancer patients using whole body hyperthermia (between

39.5°C and 40.5°C) with or without human lymphoblastoid interferon. They observed no statistically significant differences in serum IFN levels and NK cell cytotoxicity (Robins et al. 1989).

In contrast to IFNs, IL-2, used in this study, was equally effective in preventing and restoring thermal injuries on cytotoxicity of NK cells, irrespective of whether applied before, during, or after thermal treating. The effects were dose dependent. IL-2 in a concentration of 450 IU/ml elevated the cytotoxicity over non-heated controls when used before, during, or after heating. The activation of cytotoxicity was strongly dependent on the IL-2 concentration and incubation time. Kappel et al. reported similar results from heat-exposed volunteers. However, they also reported IFNα to be effective in this study setting (Kappel et al. 1991).

The combination of radiotherapy and hyperthermia has been discussed for decades (Field and Bleehen 1979). Today, knowledge of thermoradiotherapy and thermoradiobiology is deeper, and hyperthermia is used clinically as a thermal sensitizer of radiotherapy (Datta et al. 2016). Radiotherapy induces sublethal damages in tumor cells. Hyperthermia is used to inhibit reparation of the damages. In daily clinical setting, radiation therapy is used either before or after the thermal treatment (Lauber et al. 2015). Van der Zee et al. reported significantly more complete responses and longer duration of responses in pelvic cancer patients using radiation first and then hyperthermia (van der Zee et al. 2000, Jones et al. 2005). In this study, a fixed radiation dose and heating at 42°C for different times were used in both ways. No statistically significant differences were observed in the NK cell cytotoxicity.

Radiation and hyperthermia were combined in this study to examine whether the damage they caused to NK cell cytotoxicity could be prevented or repaired with IL-2. The NK cells were subjected to the combinations of IL-2, radiation, and hyperthermia or radiation, heat shock, and IL-2 treatments. A significant improvement in cytotoxicity was observed when IL-2 was applied before radiation and thermal treatment. A similar set-up of in vitro or in vivo studies was not found in the literature.

NK cells were characterized in the 1970s (Kiessling et al. 1975). Since then, they have been intensively studied in vitro and in vivo. However, systematic studies comparing effects of enrichment methods on viability and cytotoxicity of NK cells have been missing. Radiation effects on NK cells have been studied since the 1980s. The radiation dose range has been limited. Radiobiological parameters have very seldom been determined (Brovall and Schacter 1981) and fractionated

radiation effects have rarely been studied in vitro. The effects of interferons and IL-2 on irradiated and non-irradiated NK cells have been studied more extensively (Cheng et al. 2013, McDowell et al. 2015). However, systematic in vitro research using a wide range of concentrations of IL-2 and IFNα, β, and γ in preventing and restoring NK cell functions has been lacking. Also lacking have been investigations concerning radiobiological parameters of IL-2 and IFNα, β, and γ treated NK cells. In addition, systematic studies of wide-range IL-2 and IFNα, β, and γ on thermal-treated NK cell cytotoxicity are not available. The same is true for combinations of radiation and hyperthermic treatments.

In this series of studies, the issues above are investigated. Unfortunately, the accuracy of results is affected by the wide inter-individual variation of NK cell characteristics studied. Furthermore, the methods used to study a certain feature of NK cells sometimes give different results. Moreover, only one target cell type, K562, and recombinant cytokines IFNα, β, γ, and IL-2 are examined. Studies with different target cells and different cytokines have not yet been undertaken. When considering applying in vitro results in vivo therapies, the immense complexity of the immune system and the changes in tumor cells and their microenvironment also have to be taken into account.

7 Conclusions

The effects of irradiation, hyperthermia, and IFNs as well as IL-2 on viability and cytotoxicity of NK cells were studied in vitro. Several purification methods were scanned to obtain a sufficient number of usable NK cells for reliable studies. Multiple methods to measure the viability and cytotoxicity were used and compared. Irradiation used varied from low to very high non-therapeutic doses to cover the whole range of radiation effects for radiobiological calculations. The thermal range used for NK cell cytotoxicity studies ranged from hypothermia of 31°C to hyperthermia of 45°C. The effects of IFNα, β, and γ as well as IL-2 to prevent and restore the damages done by irradiation and thermal treatments were examined. The various combinations of irradiation, thermal treatments, IFNs, and IL-2, and their effects on NK cell cytotoxicity, were studied.

The main findings were the following:

1. The selection of proper NK cell enrichment methods is important for the viability and cytotoxicity of NK cells. The Dynal isolation kit was seen to be most suitable for enrichment of non-selected NK cells and the MACS isolation kit for highly purified $CD56^+$ and $CD16^+$ NK cells.

2. The standard radiobiological parameters α and β of the linear-quadratic model and the mean inactivation dose taken as the area under the curve (AUC), as well as the single-hit multi-target models, described different parts of the radiation dose-effect curve.

3. The viability and cytotoxicity of irradiated NK cells corresponded to the acutely responding tissues, the α value being relatively high and the β value low.

4. Fractionated radiation had variable effects on the viability and cytotoxicity of NK cells.

5. IL-2 increased the viability and cytotoxicity of irradiated NK cells in a concentration-dependent manner when used either before or after irradiation. The IFNs showed no dose- or schedule-dependent effects and were not able to restore viability and cytotoxicity of irradiated NK cells.

6. The cytotoxicity of NK cells showed no significant changes at temperatures between 31°C and 41°C. The most dramatic decrease in cytotoxicity was observed between 41°C and 42°C. The combinations of radiation and hyperthermia and vice versa showed no difference in damaging the NK cell cytotoxicity.

7. IL-2 was able to restore NK cell cytotoxicity in a concentration and incubation time–dependent manner when used either before, during, or after the thermal treatment. The IFNs showed no dose- or schedule-dependent effects.

8. IL-2 was even able to recover damages on cytotoxicity done by the combination of radiation and hyperthermia. IL-2 was more powerful when used before this combination treatment.

9. NK cells are important in destroying cancer cells in vivo. The in vitro effects of irradiation, IFNα, β, and γ as well as IL-2 and hyperthermia on NK cell cytotoxicity and viability can be compared with corresponding results in vivo. The usual daily radiation dose of 2 Gy does not damage but enhange the NK cytotoxicity in vitro and in vivo, as does fever-range hyperthermia. IL-2 is known as a potent NK cell-activating agent in vitro and in vivo.

10. From a theoretical point of view, it would be possible to treat cancer patients using a combination of radiation, local, or whole body hyperthermia and IL-2. As of now, no such treatment results have

been published or recorded in the clinical trials.gov file (https://clinicaltrials.gov/).

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Post-irradiation viability and cytotoxicity of natural killer cells isolated from human peripheral blood using different methods

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ABSTRACT

Purpose We compared the pre- and post-irradiation viability and cytotoxicity of human peripheral natural killer cell (NK) populations obtained using different isolation methods.

Material and methods Three methods were used to enrich total NK cells from buffy coats: (I) a Ficoll-Paque gradient, plastic adherence and a nylon wool column; (II) a discontinuous Percoll gradient; or (III) the Dynal NK cell isolation kit. Subsequently, $CD16^+$ and $CD56^+$ NK cell subsets were collected using (IV) flow cytometry or (V) magnetic-activated cell sorting (MACS) NK cell isolation kits. The yield, viability, purity and cytotoxicity of the NK cell populations were measured using trypan blue exclusion, flow cytometry using propidium iodide and ^{51}Cr release assays after enrichments as well as viability and cytotoxicity after a single radiation dose.

Results The purity of the preparations, as measured by the $CD16^+$ and $CD56^+$ cell content, was equally good between methods I–III ($p = 0.323$), but the content of $CD16^+$ and $CD56^+$ cells using these methods was significantly lower than that using methods IV and V ($p = 0.005$). The viability of the cell population enriched via flow cytometry (85.5%) was significantly lower than that enriched via other methods (99.4–98.0%, $p = 0.003$). The cytotoxicity of NK cells enriched using methods I–III was significantly higher than that of NK cells enriched using methods IV and V ($p = 0.000$). In vitro the NK cells did not recover cytotoxic activity following irradiation. In addition, we detected considerable inter-individual variation in yield, cytotoxicity and radiation sensitivity between the NK cells collected from different human donors.

Conclusions The selection of the appropriate NK cell enrichment method is very important for NK cell irradiation studies. According to our results, the Dynal and MACS NK isolation kits best retained the killing capacity and the viability of irradiated NK cells.

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Introduction

Natural killer (NK) cells were originally characterized by their capacity to mediate non-major histocompatibility complex-restricted cytotoxicity against different target cells without any priming (Kiessling et al. 1975). Today, NK cells are known to be a subset of cytotoxic innate lymphoid cells (ILC) (Spits and Di Santo 2011), a phenotypically and functionally heterogeneous group of lymphocytes representing approximately 10–15% of peripheral blood lymphocytes (PBL). Morphologically, these cells are defined as large granular lymphocytes (LGL) (Timonen et al. 1981). Phenotypically, they are characterized by the absence of CD3 expression (T cells) and the presence of CD56 and NKp46 expression and are therefore defined as $CD3^-CD56^+NKp46^+$ lymphocytes. $CD3^-CD56^{bright}NKp46^{bright}$ cells produce cytokines and chemokines and perform immunoregulatory functions. They exhibit a relatively weak capacity to kill spontaneously targeted cells, whereas $CD3^-CD56^{dim}NKp46^+$ cells are more cytotoxic (Caligiuri 2008). $CD56^{bright}$ NK cells mature into $CD56^{dim}$ NK cells (Cooper 2001).

A subset of $CD56^{dim}$NK cells expresses the NK-activating receptor CD16 (FcγRIIIa). $CD56^{dim}CD16^+$ NK cells produce low levels of cytokines. However, they exhibit natural cell-mediated cytotoxicity and strong antibody-dependent cell-mediated cytotoxicity (ADCC) (Lanier et al. 1983, Takahashi et al. 2007). Origin, development and subclasses of NK cells are defined phenotypically and functionally (Freud and Caligiuri 2006, Vacca et al. 2011).

NK cell activity is controlled by stimulatory and inhibitory factors (Bryceson et al. 2006). Continuing research has shown that the definition of NK cells and their relationship to other lymphocytes, e.g., T cells, are extremely complex (Walzer et al. 2007).

NK cells are able to distinguish abnormal cells from healthy cells and can therefore destroy so-called 'stressed' cells, e.g., tumor cells, infected cells and physically or chemically injured cells (for a review, see Vivier et al. 2012). Their capacity to kill tumor cells has led to the clinical use of NK cells for treating hematopoietic and solid malignancies (Shi et al. 2008, Terme et al. 2008, Soukup and Wang 2015).

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Radiotherapy is an important form of local therapy for cancer. However, it impairs NK activity (Dean et al. 1978). The radiation sensitivity of NK cells is controlled by co-dominant X-linked genes and radiosensitivity varies remarkably between individuals (Brovall and Schacter 1981, Schacter et al. 1985). Radiation primarily inhibits the post-binding stage of NK cell-mediated target cell lysis (Rana et al. 1990). Radiation effects on different properties of NK cells have been extensively studied both in vivo and in vitro. In our preliminary works we studied in vitro radiation effects on freshly isolated NK cells. We observed that the results of experiments were influenced by a variety of factors. We found differences in the viability and the cytotoxicity of NK cells enriched using different methods. To our knowledge, no published report has compared the radiation sensitivity of human peripheral NK cells collected using different enrichment methods. We aimed to determine the influences of irradiation on the viability and the killing capacity of human NK cell populations freshly isolated using different methods by assessing NK cell survival and cytotoxic activity. Our aim was to obtain more reliable and useful results for further radiobiological studies.

Materials and methods

NK cell enrichment

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats remaining from the processing of blood collected from healthy blood donors. Buffy coats were obtained from the Finnish Red Cross and handled anonymously. The ethical committee of the Finnish Red Cross Blood Service approved of the use of these samples for this project according to Finnish law. Before blood donation, the donors were informed that blood samples that were not required for patient treatment could be used anonymously for research if permission from the Finnish Red Cross Blood Service was obtained (www.redcross.fi). Buffy coats were prepared on the day of use. The starting materials used to enrich human peripheral NK cells were buffy coats (50 ml). The isolation scheme is shown in Figure 1.

NK cell population enrichment methods

We used three different methods to isolate total NK cells (methods I–III) and used two methods to purify the $CD16^+$ and $CD56^+$ NK cell subsets (methods IV and V).

Method I. We used a method modified from Saksela et al. (1979) to enrich NK cells from PBMC. Briefly, the PBMC were isolated using a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) as previously suggested by Böyum (1968). The resulting cells were incubated in 75 cm^2 Falcon flasks (BD Biologicals, MA, USA) for 1 h. The cells that did not adhere to the plastic were filtered through a nylon wool column (Cellular Products, NY). The non-adherent PBL were eluted in pre-warmed medium and subjected to further enrichment steps and experiments.

Method II. We used a discontinuous Percoll density gradient as proposed by Timonen and Saksela (1980). Briefly, we isolated non-adherent PBL from buffy coats as described for method I. The cells were subsequently centrifuged through a discontinuous Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient (steps of 2.5% from 50% [bottom] to 37.5% Percoll [top]) in 15 ml conical tubes (Sarstedt, Nümbrecht, Germany). The tubes were centrifuged at 300 *g* for 45 min at room temperature, and the fractions were collected from the top. Most of the NK cells were found in fractions 1 and 2.

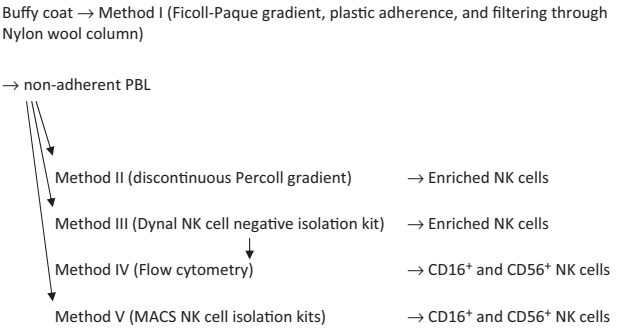

Figure 1. Isolation schemes used to enrich human NK cells from buffy coats.

Method III. Here, we also used non-adherent PBL isolated using method I. The cells were further processed with a Dynal NK cell negative isolation kit, which magnetically depletes non-NK cells from the sample, according to the manufacturer's instructions (Dynal Biotech ASA, Oslo, Norway) (Kai et al. 2004). The resulting NK cells were used in subsequent experiments.

Method IV. We used a flow cytometer (FACS Aria, BD Biosciences, CA, USA) to separate the $CD16^+$ and $CD56^+$ subsets from pre-enriched NK cells obtained using method III (Dynal isolation kit) to increase the purity of the NK cells. The antibodies used were a R-phycoerythrin (r-PE)-conjugated mouse anti-human $CD16^+$ monoclonal antibody and an allophycocyanin (APC)-conjugated mouse anti-human $CD56^+$ monoclonal antibody (BD Pharmingen, CA, USA) as described previously (Patrikoski et al. 2014).

Method V. Non-adherent PBL were isolated as described in method I. The $CD16^+$ and $CD65^+$ NK cell subsets were purified via magnetic depletion of non-NK cells and positive selection using magnetic-activated cell sorting (MACS) $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ NK Cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (Deniz et al. 2002). In this study, $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ NK cells are referred to as $CD16^+$ and $CD56^+$ NK cells, respectively.

Irradiation

In the early phase of our radiation experiments, the enriched NK cell populations were gamma-irradiated in a ^{137}Cs device (Gammacell 2000, Mølsgaard, Denmark) at a dose rate of 4.1 Gy/min at room temperature (20°C). The cells were irradiated in 5 ml snap-cap U-bottom plastic tubes (BD Falcon, NJ, USA) containing 1.25×10^6 stirred and well-oxygenated NK cells in 1 ml of medium. The gamma-irradiation dose was controlled via lithium fluoride thermoluminescence dosimetry.

In the later phase of our radiation experiments, irradiation was performed using a clinical radiotherapy accelerator (Varian

TrueBeam STx, Varian Medical Systems Inc., Palo Alto, CA) using a 6 MV photon beam and a dose rate of 6 Gy/min. The cells were irradiated at a uniform dose in 2 ml U-bottom Eppendorf ampoules (Sarstedt, Nümbrecht, Germany) containing 1 ml of medium immersed in the water phantom at 20°C.

For the dose calculation, the water phantom containing the plastic tubes was imaged using a computed tomography (CT) scanner (Toshiba Aquilion LB 1, Toshiba Medical System, Tokyo, Japan) at a slice thickness of 3 mm. The monitor units needed for the reference dose were calculated using an analytical anisotropic algorithm (AAA) (Eclipse™, v. 10.0, Varian Medical Systems Inc., Palo Alto, CA). The γ-rays from the ^{137}Cs device and the X-rays from the clinical radiotherapy accelerator have similar dose equivalent factors (Table I).

The daily radiation doses used during radiation therapy typically range from 1.8–4 Gy, and in stereotactic therapy the dose can be as high as 25 Gy. We used this dose range and non-therapeutic doses of up to 100 Gy to study the entire dose-response range of post-irradiation viability. Cell survival was assessed using the trypan blue exclusion method at 2, 6, 18 and 42 h after irradiation. For the cytotoxicity studies, doses from 0–40 Gy were selected, representing the linear portion of the semi-logarithmic dose-response curve.

Cytotoxicity assays

The cytotoxicity of NK cells was measured using the modified ^{51}Cr release assay described by Timonen et al. (1981). Briefly, we used $Na_2{}^{51}CrO_4$- (PerkinElmer Health Sciences, Groningen, The Netherlands) labeled K-562 target cells (Lozzio and Lozzio 1975) at an effector:target ratio of 12.5:1, which was selected after optimization in pilot studies. The experiments were performed in triplicate in a 96-well round-bottomed microtiter plate (Falcon BD, NJ). The plates were incubated overnight in a humidified atmosphere at 37°C. The supernatants were harvested and measured in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The relative cytotoxicity was calculated using the following formula:

$$\mathrm{Cx}(\%) = ((\mathrm{exp.} - \mathrm{spont.})/(\mathrm{max.} - \mathrm{spont.}) \times 100),$$

where exp. = average of the experimental wells, spont. = spontaneous release and max. = maximal release from K-562 cells lysed with 1% Triton X-100. The mean value of the triplicate samples was recorded. Spontaneous release was usually less than 10% of maximal release. The results were expressed relative to the non-irradiated controls.

In some cases, a single radiation dose of approximately 40 Gy or higher resulted in the lysis of a portion of the NK cells. In these cases, less ^{51}Cr was released from the target K-562 cells than spontaneous release, generating a negative result on the ^{51}Cr release assay. In such cases, the negative values were replaced with zeros in further calculations.

Trypan blue assay

To assess dead cells, we used a modified trypan blue exclusion assay described by Pappenheimer (1917). To perform the post-irradiation assay, 0.1 ml of effector cells irradiated with different doses was added to each well of round-bottomed microtiter plates (NUNC, Roskilde, Denmark) in combination with 0.1 ml of target K-562 cells at an effector:target ratio of 12.5:1. The plates were incubated at 37°C in a humidified atmosphere for 12–18 h. Next, 0.1 ml of 0.2% trypan blue was added and the plates were incubated for 10 min. The blue (dead and damaged) effector NK cells and target K-562 cells were counted under a microscope.

Table I. Comparison of radiation effects on cytotoxicity of NK cells using ^{137}Cs device and a clinical radiotherapy accelerator.

	^{137}Cs device		Accelerator		
Radiation dose (Gy)	Mean (*n*)	SD	Mean (*n*)	SD	Significance *p*
0	100 (4)	0	100 (7)	0	1.000
10	98.4 (11)	22.4	88.2 (6)	31.4	0.590
20	56.7 (11)	36.2	41.3 (7)	30.2	0.810
30	14.4 (5)	6.9	36.4 (7)	33.3	0.530
40	18.8 (7)	24.3	32.4 (6)	33.5	0.090

NK cells were isolated with method II and the mean cytotoxicity shown at different dose levels. There were no significant differences between effects on ^{51}Cr release assays at the dose levels. *n*, number of experiments.

Propidium iodide assay

Viability was also measured by flow cytometry using propidium iodide (PI) (Sigma Chemical Co., MO, USA) as described previously (Malygin et al. 1994). Briefly, isolated lymphocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies and PI was added. The percentage of PI-stained cells, i.e. dead cells, was measured using flow cytometry based on the red fluorescence, while viable lymphocytes exhibited green fluorescence.

Culture conditions

The cells were cultured and the experiments were conducted at 37°C in a humidified air atmosphere containing 5% CO_2. RPM 1640 medium (Orion Diagnostica, Vantaa, Finland) was supplemented with 10% heat-inactivated fetal calf serum (FCS, Flow Laboratories, Irvine, Scotland, UK), L(+) glutamine (0.3 g/l) (Fluka, Buchs, Switzerland) and gentamycin (20 μg/ml) (Flow Laboratories, Irvine, Scotland, UK) and this combination is referred to as 'medium' throughout this report.

Statistical analysis

The data were analyzed using SPSS 15.0 software (SPSS Inc., Chicago, IL). The results are presented as the means ± standard deviation (SD) of several independent experiments. Statistically significant differences between the group means were assessed via one-way analysis of variance (ANOVA). Post hoc analysis was performed using Bonferroni's modification of the *t*-test. The Kruskal-Wallis test and the Mann-Whitney U test were used for data that could not be assessed via ANOVA (e.g., data that were not normally distributed). The constituent ratios were compared using the χ^2 test (Chi-square test) and the Fisher exact test. Differences were considered to be significant at $p < 0.05$ (2-sided). Linear regression analysis was used to investigate the relationships between the variables. Residual

Table II. The yield of NK cells and the percentage of the $CD16^+$ and $CD56^+$ subsets enriched using methods I–V.

Method (*n*)	Output/10^6 non-adherent PBL	NK subsets as percentage of PBL mean ± SD	$CD16^+$ and $CD56^+$ NK cells isolated/10^6 PBL
Method I (28)			
$CD16^+$	NA	31.4 ± 24.2	0.3 ± 0.2
$CD56^+$		15.4 ± 9.5	0.2 ± 0.1
Method II (13)			
$CD16^+$	0.6 ± 0.6*	39 ± 16.5	0.2 ± 0.2***
$CD56^+$		23.9 ± 16.5	0.1 ± 0.1
Method III (31)			
$CD16^+$	0.2 ± 0.1	42.7 ± 30.9	0.2 ± 0.2
$CD56^+$		36.4 ± 35.3	0.1 ± 0.1
Method IV (10)			
$CD16^+$	0.1 ± 0.1	100**	0.1 ± 0.1
$CD56^+$	0.2 ± 0.1	100**	0.1 ± 0.1
Method V (7)			
$CD16^+$	0.3 ± 0.3	99.5 ± 0.4**	0.3 ± 0.3****
$CD56^+$	0.3 ± 0.4	97.7 ± 2.5**	0.3 ± 0.3****

For harvesting non-selected NK cells, method II was the most effective (*$p = 0.012$). For harvesting highly purified NK cells, methods IV and V were equally effective. The percentage of $CD16^+$ and $CD56^+$ NK cells did not significantly differ between methods I–III and IV–V. Methods IV and V resulted in significantly higher enrichment of these subsets than methods I-III (**$p = 0.005$). Method II yielded more $CD16^+$ cells than method III (***$p = 0.033$). Method V yielded the highest number of $CD16^+$ and $CD56^+$ cells combined (****$p = 0.009$). Number of experiments (*n*) indicates numbers of independent experiments made for NK cell yields.

statistics were controlled. Non-parametric correlations were calculated using Spearman's rho analysis.

Results

Enrichment of NK cell populations

The mean output from one buffy coat was $144.3 \pm 143 \times 10^6$ non-adherent PBL. Significant differences in the cell yields were observed for buffy coats from different donors, and this difference displayed a very high standard deviation. The effectiveness of different enrichment methods was measured as the output of 10^6 cells per input of 10^6 non-adherent PBL from one buffy coat. The yield of non-selected NK cells using method II was significantly higher than that using method III ($p = 0.012$). For highly purified NK cells, methods IV and V were equally effective at yielding $CD56^+$ ($p = 1.000$) and $CD16^+$ ($p = 0.151$) cells (Table II).

NK cell purity in enriched populations

The purity of the cell populations isolated using different methods was assessed using flow cytometry following anti-human $CD16^+$ and $CD56^+$ antibody staining. Other NK cell subclasses were not evaluated. The percentages of $CD16^+$ and $CD56^+$ NK cells in the enriched NK cell population obtained using methods I–V are presented in Table II. Methods I–III equivalently enriched non-selected NK cells ($p = 0.323$). Methods IV and V isolated these cells more effectively ($p = 0.005$), yielding approximately 100% purity.

Yields from the enrichment methods

Method II produced significantly more $CD16^+$ cells than method III ($p = 0.033$) (Table II). The yield of $CD56^+$ cells was equivalent between these two methods ($p = 1.000$). Methods IV and V were equally effective at harvesting $CD16^+$ ($p = 1.000$) and $CD56^+$ cells ($p = 1.000$). Relative to the total number of NK cells ($CD16^+$ and $CD56^+$) from 10^6 PBL from one buffy coat, there were significant differences ($p = 0.000$) among methods I–V. The yield was significantly the highest for method V (method V vs. methods II, III and IV: $p = 0.009$, 0.000 and 0.069, respectively).

Viability of NK cells isolated using methods I–V

The viability of the cell populations collected using methods I–V was assessed based on trypan blue exclusion (Table III). The viabilities of the cells isolated using methods I–III and V were identical ($p = 1.000$) and were greater than 95%, whereas the viability of the cells isolated using method IV (flow cytometry) was significantly lower than that of the cells isolated using any other method ($p < 0.023$). These results indicated that flow cytometric cell sorting may reduce NK cell viability.

Cytotoxicity of isolated human NK cell populations

Table IV shows the cytotoxicity of isolated non-irradiated NK cell populations, as measured by the ^{51}Cr release assay. Two groups with different degrees of cytotoxicity were observed: (1) The non-selected NK cells enriched using methods I–III were equally cytotoxic ($p = 0.224$); (2) the highly purified $CD16^+$ and $CD56^+$ NK cells (using methods IV and V) displayed equivalent killing capacity ($p = 0.471$). However, the NK cells isolated using methods IV and V were significantly less cytotoxic than those isolated using methods I–III ($p = 0.000$).

The $CD16^+$ cells isolated using methods IV and V exhibited identical levels of cytotoxicity ($p = 0.662$). The same result was obtained for $CD56^+$ cells ($p = 1.000$). Therefore, for the following experiments, the results from the $CD16^+$ and $CD56^+$ NK cells isolated using methods IV and V were combined. There were no differences in cytotoxicity between the combined $CD16^+$ and $CD56^+$ cells and either NK cell subset alone ($p = 1.000$).

The cytotoxicity of non-irradiated NK cells purified using different methods was also measured by counting dead K-562 target cells using the trypan blue method. No statistically significant differences were observed between the NK cell groups ($p = 0.820$).

Effects of irradiation on the survival of an enriched human NK cell population as a function of dose and time

The effects of radiation on non-selected NK cells were evaluated using the trypan blue exclusion and propidium methods, in whose viability was considered as an endpoint. The cell population enriched using method II was selected because its cell output was higher and its purity was statistically comparable to that the other non-selected NK cell preparations.

NK cells were irradiated with single doses ranging from 0–80 Gy. The viability data measured using both trypan blue and

Table III. The viability of NK cell populations enriched using methods I–V as determined by the trypan blue exclusion assay.

Method (*n*)	Viability (%) ± SD
Method I (25)	98.6 ± 1.8
Method II (35)	99.4 ± 0.7
Method III (33)	98.0 ± 2.3
Method IV (12)	85.5 ± 26.3*
Method V (16)	96.2 ± 3.0

*This method resulted in significantly lower viability than the other methods ($p < 0.023$). The results are expressed as the percentage of cells counted and the standard deviation is indicated (± SD). *n* indicates number of independent experiments.

Table IV. Cytotoxicity of non-irradiated human NK cells isolated using methods I–V, as determined by the ^{51}Cr release assay.

Method (*n*)	Cytotoxicity (%, mean ± SD)
Method I (6)***	31.6 ± 19.8*
Method II (7)***	46.9 ± 18.6*
Method III (20)***	32.9 ± 18.9*
Method IV***	
$CD16^+$ (5)	13.2 ± 13.2**
$CD56^+$ (6)	18.1 ± 9.5**
Method V***	
$CD16^+$ (6)	15.6 ± 13.7**
$CD56^+$ (4)	18.1 ± 8.9**

The relative cytotoxicity of several independent experiments was calculated. Number of experiments (*n*) is presented. The NK cells isolated using methods I–III were equally cytotoxic (*$p = 0.224$). No difference in the cytotoxicity of $CD16^+$ and $CD56^+$ cells was detected between the use of methods IV and V (**$p = 0.471$). NK cells isolated using methods IV and V were significantly less cytotoxic than those isolated using methods I–III (***$p = 0.000$).

Table V. The comparison between trypan blue exclusion and propidium iodide methods to describe cell survival of irradiated NK cells.

	Propidium iodide		Trypan blue		Significance
Radiation dose (Gy)	Mean (*n*)	SD	Mean (*n*)	SD	*p*
0	100 (3)	0	100 (4)	0	1.000
10	75.5 (2)	7.8	65.0 (4)	13.9	0.530
20	61.7 (3)	16.2	51.8 (3)	7.8	0.700
30	46.5 (2)	26.2	36.5 (2)	0.0	1.000
40	46.3 (3)	10.5	18.6 (4)	16.1	0.110
80	32.7 (4)	8.1	13.0 (4)	4.3	0.100

Non-selected NK cells were irradiated with doses from 0–80 Gy. The effect on viability as endpoint was evaluated using propidium iodide and trypan blue methods. There were no statistically significant differences between the methods used at the dose levels. *n*, number of experiments.

propidium iodide methods at all dose levels were statistically equal (Table V).

The survival of NK cells was followed over a period of 42 h using trypan blue exclusion method. It decreased as a function of dose and time after irradiation (Figure 2). However, even at the highest dose of 100 Gy, at 2 h post-irradiation, more than 95% of the NK cells were not stained blue; e.g., the NK cells remained alive. At 2, 6, 18 and 42 h post-irradiation, the average percentage of dead cells following irradiation at a dose of 1 Gy was 1.3%, 6.1%, 20.7% and 20.2%, respectively, and that following irradiation at a dose of 10 Gy was 2.7%, 11.6%, 16.4% and 67.5%, respectively. Even at high doses (80–100 Gy), a long observation time was required for all of the NK cells to stain blue. For subsequent studies, an incubation

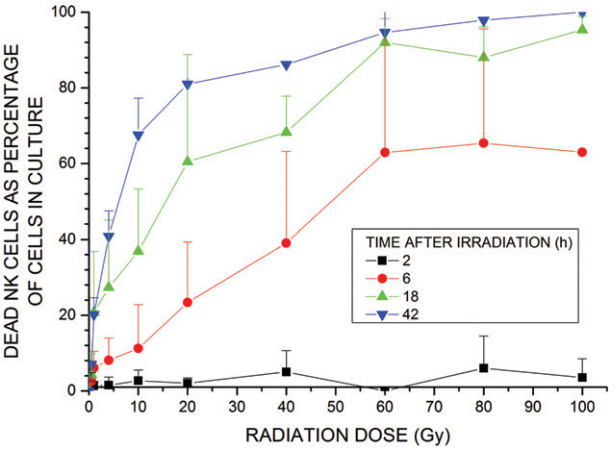

Figure 2. The death of non-selected NK cells after different single radiation doses and post-irradiation incubation periods. Non-selected NK cells were irradiated using doses ranging from 0–100 Gy. Viability of cells were evaluated using trypan blue exclusion method at various incubation time periods from 0–48 hours. Dead cells are presented as percentages of cells in cultures. Error bars represent standard deviations for at least two independent experiments.

period of 18 h was selected, and a dose range from 0–40 Gy, representing the linear portion of the dose-response curve on a semi-logarithmic scale, was used.

Effects of irradiation on the killing capacity of enriched NK cell populations

Irradiation reduced the viability of NK cells in a dose-dependent fashion. Next, the effects of irradiation on NK cells were studied using their cytotoxicity as an end-point. The cell populations enriched using methods I–V were irradiated with a single dose varying from 0–40 Gy. The cytotoxicities of the irradiated NK cell populations were measured via the ^{51}Cr release assay (Figure 3).

The cytotoxicities of the cell populations enriched using methods I–III (representing non-selected NK cells) were comparable ($p = 0.353$), and in this respect these cells were more radiation-resistant than the highly purified $CD16^+$ and $CD56^+$ NK cell subsets isolated using methods IV–V ($p = 0.000$). Combining the ^{51}Cr release results from both the $CD16^+$ and $CD56^+$ NK cells demonstrated that the cytotoxicity of these cells was significantly further inhibited by radiation than that of the cells isolated using method I or II ($p = 0.003$ and 0.000, respectively).

Also, the effect of irradiation dose levels on the cytotoxicity of NK cells enriched using methods I–V were calculated and comparisons between methods I–III and IV–V were made (Table VI). Here again, the radiation dose levels damaged the cytotoxicity of NK cells isolated by methods I–III to the same extent. The same is true for methods IV–V. The cytotoxicity of the NK cells differ significantly between methods I–III and IV–V at dose levels 10–20 Gy (Table VI).

The radiation sensitivity of NK cells was highly variable. NK cells from 16 consecutive donors were irradiated using 20 Gy. Two hours after treatment the cytotoxicity was in median 26% ranging from 3–120%.

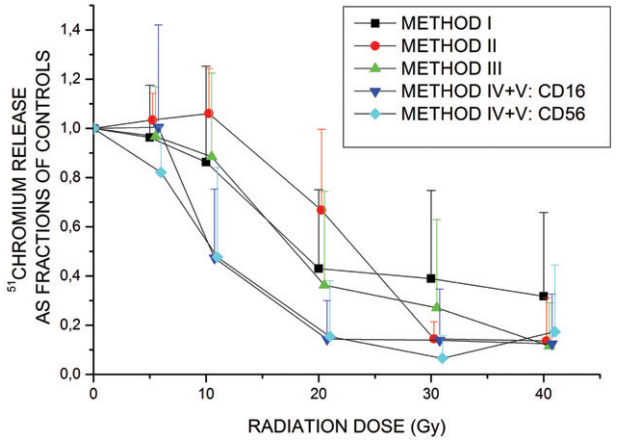

Figure 3. The cytotoxicity of NK cells enriched using methods I–V and irradiated with different single doses. The effects of irradiation on the cytotoxicity of NK cell populations were measured at 18 h after irradiation using the ^{51}Cr release assay. The dose levels were 0, 5, 10, 20, 30 and 40 Gy. Number of independent experiments for each dose levels was for method I: 6, 5, 5, 6, 6 and 6, for method II: 7, 4, 7, 6, 2 and 6, for method III: 16, 4, 11, 12, 13, and 8, for CD16$^+$ cells: 12, 4, 7, 8, 6 and 5, for CD56$^+$ cells: 10, 2, 4, 3, 5 and 4. Error bars represent standard deviations. Experiments using non-selected cell populations obtained using methods III and highly purified cells obtained using methods IV–V were performed side by side.

Table VI. Statistical significance of the differences in the radiation dose dependence of cytotoxicity between NK cells enriched using methods I–III and IV–V.

Isolation methods Radiation dose (Gy)	I–III Significance (p)	IV–V Significance (p)	I–III vs. IV–V Significance (p)
5	0.84	0.35	0.29
10	0.50	0.85	0.00
20	0.24	0.45	0.01
30	0.74	0.33	0.03*
40	0.12	0.61	0.29

NK cells enriched using methods I–V were irradiated with doses from 0–40 Gy. The significance of damages to cytotoxicity measured by ^{51}Cr release was compared at all dose levels among the methods I–III and IV–V as well as between the methods I–III and IV–V. Non-irradiated NK cells served as controls.
*At the dose level of 30 Gy the difference between methods I–III and IV–V was significant only, when method II was excluded.

As expected, reducing the survival of the irradiated NK cell population decreased its capacity to kill radioactively labeled target K-562 cells, as demonstrated by the release of ^{51}Cr from the target cells. The proportion of dead NK cells was measured by the trypan blue exclusion. In the dose range from 1–10 Gy, the proportion of dead NK cells was approximately 20–30%, whereas these cells induced a level of ^{51}Cr release, which was occasionally higher than that for the non-irradiated controls. At doses from 20–40 Gy or higher, the proportion of dead NK cells increased, and ^{51}Cr release decreased rapidly, indicating the decreased viability and cytotoxicity of the irradiated NK cells.

The Spearman's correlations were calculated between radiation dose, ^{51}Cr release, dead NK cells and dead K-562 cells. The best correlation was between radiation dose and dead NK cells, correlation coefficient being 0.921 (2-tailed). The coefficients between other variables were all between approximately 0.6 and 0.7, being positive or negative depending on combination tested (Table VII).

Radiation sensitivity of NK cell populations based on the trypan blue exclusion method

In addition, we did not find any statistically significant differences in radiosensitivity between NK cell populations enriched using methods I–V based on the trypan blue assay ($p = 0.230$). We also compared the results from the ^{51}Cr release assay with those from the trypan blue exclusion assay in NK cells enriched using method II. Both analyses were performed at the same time-point after irradiation and ^{51}Cr release assay incubation. Dead target K-562 cells were counted using the trypan blue exclusion method. The results of the ^{51}Cr release assay and the proportion of dead effector NK cells counted using the trypan blue method equally effectively described damage to NK cells caused by irradiation ($p = 0.800$) in terms of cell death and the loss of cytotoxicity. The trypan blue exclusion method was not able to detect the elevation of NK cell activity induced by low radiation doses. The proportion of dead K-562 cells was lower than the proportion of ^{51}Cr released by these cells. K-562 cells release ^{51}Cr earlier than they absorb the trypan blue dye and are thus counted as dead.

Discussion

To enrich non-selected NK cells we used older methods I–II from 1970s and 80s based on density gradient centrifucations and adherence. Method III uses magnetically labeled antibodies. All these methods harvested non-selected NK cells with sufficient yield, viability, cytotoxicity and radiation sensitivity. The method II yielded more non-selective NK cells than the Dynal NK Cell Negative Isolation Kit, whereas the purity of these populations did not differ. The MACS kit harvested highly purified CD16$^+$ and CD56$^+$ NK cell subsets with equal purity but higher yield and viability than flow cytometry.

To assess the viability of irradiated NK cells the trypan blue and propidium iodide methods were used. At all dose levels both methods were equally effective to describe cell death as endpoint.

The viability of NK cells enriched using flow cytometry was lower than those enriched using other methods. This may be due to mechanical handling inside the flow cytometry apparatus. In addition, the cytotoxicity of the non-selected NK cell populations was significantly higher than of the highly purified CD16$^+$ and CD56$^+$ subsets. It is possible that methods IV–V, which were performed to enrich for CD16$^+$ and CD56$^+$ cells, are more damaging to cells than methods I–III. We did not find any notes on these findings in the literature. It is also possible that NK cell cytotoxicity requires the activity of several NK cell subsets.

Following exposure to low radiation doses, NK cell cytotoxicity was higher than that of the non-irradiated controls. The effects of low dose irradiation on living cells have been extensively studied including among other aspects of radiation-induced hormesis, radioresistance, adaptive responses and bystander effect. For references, see Rodel et al. (2012), Tang and Loke (2015). Regarding NK cells, previously it has been proposed to occur because NK suppressor cells have

Table VII. Correlations between radiation dose, ^{51}Cr release, dead NK and dead K-562 cells.

		Radiation dose (Gy)	^{51}Cr release	Dead NK cells	Dead K-562 cells
Radiation dose (Gy)	Correlation coefficient	1.000	−0.605**	0.921**	−0.584**
	Sig. (2-tailed)	–	0.000	0.000	0.002
	n	73	63	37	26
^{51}Cr release	Correlation coefficient	−0.605**	1.000	−0.641**	0.710**
	Sig. (2-tailed)	0.000	–	0.000	0.001
	n	63	63	35	18
Dead NK cells	Correlation coefficient	0.921**	−0.641**	1.000	−0.666*
	Sig. (2-tailed)	0.000	0.000	–	0.013
	n	37	35	37	13
Dead K-562 cells	Correlation coefficient	−0.584**	0.710**	−0.666*	1.000
	Sig. (2-tailed)	0.002	0.001	0.013	–
	n	26	18	13	26

*Correlation is significant at the 0.05 level (2-tailed);
**Correlation is significant at the 0.01 level (2-tailed). The correlations between radiation dose, ^{51}Cr release, dead NK and dead K-562 cells were calculated using Spearman's rho test. The correlation coefficients and statistical significance (Sig. [2-tailed]) are presented. *n* represents total number of compared observations.

greater radiation sensitivity than other NK cells (Dean et al. 1978). However, we detected the activation of NK cells, not only on the cytotoxicity tests, but also based on the intracellular ATP levels and even the ATP levels of K-562 cells (data not shown). The increase in ^{51}Cr release at low radiation doses may also be caused by the recruitment of $CD16^+$ and $CD56^+$ cells with low NK cell activation, or by a greater cytotoxicity on an individual NK cell basis, or both. In fact, the activation of $CD16^+$ cells (Rana et al. 1990) and T cells (Makinodan and James 1990) may be a common adaptive reaction of cells exposed to low doses of radiation.

On the ^{51}Cr release test, irradiation doses of 40 Gy or higher caused, on some occasions, less ^{51}Cr release from target K562 cells than the level of spontaneous release. Therefore, in the ^{51}Cr release cytotoxicity calculation, negative results were obtained, and the radiation dose-effect curve exhibited an upward trend. These phenomena might be due to a feeder-like effect of dead NK cells on K-562 cells.

No recovery of viability or cytotoxicity after irradiation was observed in vitro during the observation period, which might be explained by the absence of NK cell-stimulating cytokines such as interferons and interleukins. Previously, we (Hietanen et al. 1995) and Franzese et al. (2013) showed that interleukin-2 clearly reduces the effect of radiation on NK cells. However, in vivo situations after radiotherapy many cytokines could activate NK cells and help the recovery as shown in preclinical and clinical studies (McDowell et al. 2015, Shahabi et al. 2015). Thus, our results could not be directly applied to clinical behavior during cancer treatments. Our main goal was to examine radiobiological characters for future studies.

The post-radiation survival of non-selected NK cells was studied using the trypan blue exclusion method. However, even following irradiation at high doses and long observation times after irradiation, some cells did not stain blue, which may be a peculiarity of the trypan blue exclusion method or a feature of the injured cells. In addition, we have observed the same phenomenon on flow cytometry tests using propidium iodide.

Interestingly, the $CD56^+CD16^+$ and $CD56^+CD16^-$ NK cells that we isolated using either flow cytometry (method IV) or a MACS isolation kit (method V) were equally cytotoxic. According to the literature, $CD56^+CD16^+$ NK cells naturally exhibit high cell-mediated cytotoxicity, whereas $CD56^+CD16^-$ NK cells tend to play an immunoregulatory role (Nagler et al. 1989). Vokurková et al. (2010) showed that $CD56^+$ cells are more radiosensitive than $CD16^+$ cells. However, we did not detect any differences in radiation sensitivity between these NK subsets. Grzywacz et al. (2007) reported that upon co-culturing with K562 cells, some $CD56^{dim}CD16^+$ cells became $CD56^+CD16^{dim}$ or even $CD56^+CD16^-$ and, therefore, became less cytotoxic. However, this phenomenon might not fully explain our findings. It is more likely that the remarkable inter-individual variation between donors whose samples were used for this study accounts for the differences between our results and the published data. As early as the 1970s and 1980s, Santoli et al. (1976) observed considerable variation in the cytotoxicity of NK cells obtained from different donors and linked this observation to the HLA-A3-B7 haplotype. Zaretskaya et al. (1983) showed fluctuations in human NK cell activity in vitro. It is known that NK cell activity is regulated by several inhibitory and stimulatory signals (Bryceson et al. 2006). Dean et al. (1978) reported that the NK cell response to radiation is variable and complex. Our results support these previous findings.

The trypan blue exclusion method applied to the target K-562 cells was less sensitive than the ^{51}Cr release assay in detecting differences in NK cell cytotoxicity between the isolated populations. The ^{51}Cr release assay was a less laborious method for studying NK cell toxicity than the trypan blue exclusion assay. In the literature, there are very few comparisons between the ^{51}Cr release assay and the trypan blue exclusion method (Bonavida and Wright 1982). For decades, the ^{51}Cr release assay has been the 'gold standard' for measuring cell-mediated cytotoxicity. Although numerous methods have been developed to replace it (Zaritskaya et al. 2010), the ^{51}Cr release assay remains useful for large-scale radiation studies.

Considering the NK cell yield, enrichment purity, cytotoxicity and radiation tolerance, method II and the Dynal isolation kit were comparable; however, method III was more convenient for enriching non-selected NK cells. The MACS isolation kit harvested more viable highly purified NK cells than flow cytometry. In our hands both the Dynal and MACS isolation kits

were the best methods. According to our results, the selection of the purification methods and selected endpoints are highly important for future studies of the effects of irradiation on NK cells.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Author contributions

Tenho Hietanen performed the experiments. All authors (Tenho Hietanen, Maunu Pitkänen, Mika Kapanen and Pirkko-Liisa Kellokumpu-Lehtinen) participated in interpreting the results and writing the manuscript. All of the authors agreed to the contents of this manuscript.

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Effects of Single and Fractionated Irradiation on Natural Killer Cell Populations: Radiobiological Characteristics of Viability and Cytotoxicity *In Vitro*

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Abstract. *Background: Natural killer (NK) cells are important in destroying tumor cells. However, they are damaged by radiation therapy. We studied the effects of single and fractionated irradiation on the viability and cytotoxicity of human non-selected NK cells and sub-groups with cluster of differentiation (CD) CD16$^+$ and CD56$^+$ in vitro. Only very few studies dealing with the standard radiobiological parameters for characterizing NK cells exist in the literature. Materials and Methods: NK cell populations were isolated from buffy coats using different methods and irradiated with single doses up to 80 Gy and fractionated doses of 10 or 30 Gy with different numbers of applications and at different intervals. The study end-points were viability using propidium iodide (PI), trypan blue and intracellular adenosine triphosphate (ATP) assays, and cytotoxicity using the ^{51}Cr-release assay. The standard radiobiological parameters α and β of the linear-quadratic (L-Q) model and the mean inactivation dose $\bar{D}$taken as the area under the curve (AUC) were calculated to characterize the radiosensitivity of different NK cell populations. Results: The AUC values of the 51Cr release data in the dose range of 0-40 Gy were as follows: for non-selected NK cells, 23.6-20.9 Gy; for CD16$^+$ and CD56$^+$ cells, 14.5-13.2 Gy. The AUC values of ATP, trypan blue and propidium iodide methods equally well described the viability of irradiated NK cells. The α/β ratio for cytotoxicity and viability data in the L-Q model corresponded to the acutely responding tissues. Splitting a 30-Gy dose into two fractions applied at different intervals caused a significant rise in ATP levels and cytotoxicity. Dividing the total dose into four doses applied at fixed intervals also resulted in significant elevations of ATP content and cytotoxicity of NK cells at 10 Gy. Conclusion: According to the L-Q method, irradiated NK cells behaved similarly to acutely responding human tissues with respect to cytotoxicity and viability. The AUC proved very useful for comparing the effects of irradiation on NK cells.*

Natural killer (NK) cells were originally defined functionally, based on their ability to mediate non-histocompatibility-restricted cytotoxicity against different tumor target cells (1) including *de novo* (2) and existing tumor cells (3). NK cells can also control the metastatic spread of malignant cells (4). Furthermore, NK cells contribute to the natural resistance against microbial infections, the grafting of bone marrow transplants, and graft *versus* host disease, and regulate the differentiation of hematopoietic and other cells (5). NK cells represent approximately 10-15% of peripheral blood mononuclear lymphocytes. They are characterized phenotypically by possessing CD56 and NKp46 receptors, and comprise of two major sub-groups, CD56$^+$ and CD16$^+$, which have distinctive properties. They do not display the CD3$^+$ T-cell phenotype.

Autologous or allogenic NK cells, alone or activated by interleukin-2, have been used in cancer therapy (6-7). In addition to cytokines current development in therapy has brought potential drugs like lenalidomide and bortezomib, enhanced NK cell function with monoclonal antibodies, adoptive therapy using expanded and activated NK cells, and use of NK-cell chimeric antigen receptors. For recent reviews of NK cell-based immunotherapy, see McDowell *et al.* (8) and Terme *et al.* (9).

Radiotherapy, one of the most common cancer therapies, may impair NK activity (10). The radiosensitivity of NK cells

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Key Words: Natural killer cells, natural killer cell subsets, irradiation, viability, cytotoxicity, radiobiological models, enrichment methods.

is controlled by X-linked genes and varies considerably among individuals (11, 12). Radiotherapy effectively inhibits the post-binding stage of NK cell-mediated natural target cell lysis (13). On the other hand, radiotherapy has been used for immune-priming of tumor cells and tumor microenvironment. Therefore, combination of radiotherapy and immunotherapy has been investigated (14).

Only a very limited number of systematic *in vitro* studies are available to document the effects of irradiation, fractionated and single-dose schemes of irradiation, observation time after irradiation and parameters of radiobiological models (10, 15). Radiobiological effects are commonly quantified by the linear-quadratic model with two parameters, linear term α and quadratic term β, which can be determined by fitting the model to the dose–effect data (16). The model enables for the separation of radiation sensitivity for lethal and sublethal radiation injuries and facilitates categorizing the cells as early or late responders. A simpler method in the quantification of the radiosensitivity is to determine the area under the dose–effect curve (AUC) (16). The AUC is commonly used in calculations of the effects of cytotoxic drugs (17, 18).

Our aim in this study was to elucidate the radiobiological effects of single and fractionated gamma and X-ray irradiation on non-selected NK cells, as well as the $CD16^+$ and $CD56^+$ subsets. The study end-points were viability, as measured by propidium iodide (PI), trypan blue and intracellular adenosine triphosphate (ATP) assays, and cytotoxicity, as measured by the 51Cr-release assay. The standard radiobiological parameters α and β, as well as the mean inactivation dose $\bar{D}$ as the AUC, were calculated to characterize the radiosensitivity of the different NK cell populations.

Materials and Methods

Enrichment of NK cell populations. NK cells were isolated from buffy coats obtained from the Finnish Red Cross Transfusion laboratory with permission of the ethical committee of the Finnish Red Cross Blood Service (customer number 6129, approval number 331/2013, tutkijaluvat@veripalvelu.fi) in accordance to Finnish law. Before blood donation, donors were informed that blood samples that are not required for patient treatment can be used anonymously for research work. Buffy coats were prepared on the day of use.

Non-selected and highly purified $CD56^+$ and $CD16^+$ NK cell populations were enriched using different methods as described previously (submitted to Int J Radiat Biol by Hietanen T., Pitkänen M., Kapanen M., Kellokumpu-Lehtinen P-L., 2015). In brief, we used Ficoll-Paque (method I) and Percoll gradient centrifugation (method II) and a Dynal NK cell negative isolation kit (Dynal Biotech ASA, Oslo, Norway) (method III). The $CD16^+$ and $CD56^+$ subgroups were isolated using flow cytometry (method IV) and magnetic depletion of non-NK cells and positive selection using MACS $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ CD16- NK cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) (method V). The purity of preparations was confirmed by Giemsa-stained cytocentrifuge preparations and flow cytometry.

Flow cytometric assay. For the phenotypic analysis of enriched NK cells, we used R-phycoerythrin-conjugated mouse monoclonal antibody to human $CD16^+$ and allophycocyanin-conjugated mouse monoclonal antibody to human $CD56^+$ (BD Pharmingen, San Diego, CA, USA) as described elsewhere (19). The cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Irradiation. The cells were irradiated in an early phase of the study using a ^{137}Cs device (GAMMACELL 2000, Mølsgaard Medical, Risø, Denmark) and later with a clinical radiotherapy accelerator (Varian TrueBeam STx, Varian Medical Systems Inc., Palo Alto, CA, USA) as described in our previous work (submitted to Int J Radiat Biol by Hietanen *et al.* 2015). In viability studies, the radiation dose ranged from 0 Gy to the non-therapeutic dose of 80 Gy to explore the whole dose–response curve for radiobiological calculations. The effects on cytotoxicity were investigated in the dose range from 0 to 40 Gy, according to our previous study (submitted to Int J Radiat Biol by Hietanen *et al.* 2015).

In the split-dose studies, doses of 10 and 30 Gy were selected from the upper and lower linear part of the semi-logarithmic part of the dose–response curve. These doses were split into two, with each dose divided into two fractions applied at intervals from 0 to 24 hours, or split up to three equal fractions applied at 3-hour intervals.

Assessment of viability. Viability was measured by flow cytometry using PI (Sigma Chemical Co., St Louis, MO, USA) as described previously (20). Briefly, isolated lymphocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for 30 minutes on ice, washed, and then PI was added at a final concentration of 4 μM. The percentage of PI-stained cells, *i.e.* non-viable cells, was measured using flow cytometry based on the red fluorescence, while viable lymphocytes exhibited green fluorescence.

In the trypan blue exclusion method (21), 0.1 ml of the NK cell suspension and 0.1 ml of 0.2% trypan blue solution were mixed. After 10 minutes, 200 cells were examined under a microscope, and all blue and damaged cells were counted as dead.

Commercial kits from LKB Wallac (Turku, Finland) were used for the intracellular ATP method (22) according to the manufacturer's instructions. The amounts of ATP were then measured using an LKB Wallac 1251 luminometer, and the results were obtained as millivolts and expressed as values normalized to those of non-irradiated controls. Viability assays were performed at different times, from 0 to 72 hours post-irradiation.

Cytotoxicity test. The cytotoxicity of enriched cell populations was measured by the modified ^{51}Cr release assay described by Timonen *et al.* (23) using K-562 cells (LGC Standards GmbH, Wesel, Germany) as a target. The relative cytotoxicity was calculated using the following formula: Cx (%)=((exp.-spont.)/(max.-spont.)) where exp is experimental release; spont. is spontaneous release, and max. is maximal release from K-562 cells lysed with 1% Triton X-100. The spontaneous release was less than 10% of the maximal release. The effector-to-target ratio (E:T) of 12.5:1 used for the cytotoxicity studies was based on pilot studies. The cytotoxicity at this ratio was 52.1±11.5% (SD) of the maximal release. The results were normalized to those of the controls.

The characteristics of the isolated non-selected NK cell populations, including the viability, purity and cytotoxicity, and those of the $CD16^+$ and $CD56^+$ subsets, were presented in our

Table I. *Linear-quadratic model parameters of the cytotoxicity data of the irradiated natural killer cells obtained using the ^{51}Cr-release method (dose range from 0 to 40 Gy). The cytotoxicity test was performed 18 h post-irradiation. Standard errors of the parameters (±SE) and p-values are given. CD16$^+$ enriched with methods IV and V were combined, as well as CD56$^+$ cells.*

Enrichment method	α (Gy^{-1})	± SE	β (Gy^{-2})	±SE	*p*-Value
Method I	0.03199	0.008	–2.57E-05	0.000	0.000
Method I *	0.0313	0.003	0	0	1
Method II	0.01362	0.030	0.00164	0.001	0.002
Method III	0.01532	0.001	0.00106	0.000	0.000
CD16$^+$	0.08985	0.029	–7.55E-04	0.001	0.009
CD16$^+$ *	0.0732	0.011	0	0	1
CD56$^+$	0.0885	0.032	–3.66E-04	0.001	0.008
CD56$^+$ *	0.0807	0.011	0	0	1

*When β was negative and assigned a value of zero, a new α value was re-determined.

Table II. *Area under the survival curve (AUC) values calculated for the ^{51}Cr-release data of natural killer cells 18 h post-irradiation.*

Enrichment method	AUC (Gy)
Method I	23.6
Method II	24.1
Method III	20.9
Methods IV+V: CD16$^+$	14.5
Methods IV+V: CD56$^+$	13.2

previous paper (submitted to Int J Radiat Biol by Hietanen *et al.* 2015). CD16$^+$ cells enriched using methods IV and V were equally cytotoxic. The same is also true for CD56$^+$ cells. Therefore, we combined the CD16$^+$ cells and CD56$^+$ cells for further cytotoxicity analyses. These sub-populations were called CD16$^+$ and CD56$^+$, respectively.

Calculation of the radiobiological parameters. The following standard mathematical radiobiological models were used to quantify the radiation dose–response curves: I: The linear-quadratic model $S=e^{-(\alpha D+\beta D^2)}$, where S represents the surviving fraction, and D is the radiation dose in Gy (24). The linear-quadratic model has two inactivation parameters, the linear term α (Gy^{-1}) and the quadratic term β (Gy^{-2}). When negative values were obtained for β, and thus biologically meaningless, the negative values were assigned a value of zero, and the linear-quadratic model was re-fitted as suggested by Fertil *et al.* (25). II: The mean inactivation dose $\bar{D}(Gy)=\int S(D)dD$ represents the area under the survival curve (AUC) in linear coordinate representation (16), where S(D) represents the survival probability and D the radiation dose. The AUC was not calculated from the fitted linear-quadratic model but by direct integration of the dose–response curve in question.

The two models were fitted to the experimental data using the OriginPro 2015 program (OriginPro Corporation, Northampton, MA, USA).

Culture conditions. Cells were cultured as described in our previous work (submitted to Int J Radiat Biol by Hietanen *et al.* 2015). The effector NK cells and target K-562 cells were cultured in RPM-1640 medium (Orion Diagnostica, Vantaa, Finland) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), L(+)-glutamine (0.3 g/l) (Fluka, Buchs, Switzerland) and gentamycin (20 μg/ml) (Flow Laboratories). The experiments were conducted at 37˚C in a humidified air atmosphere containing 5% CO_2.

Basis of data presentation and statistical analysis. For statistical calculations, the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA) was used. The results are presented as the mean±SD or standard error (SE). Analysis of variance (ANOVA) with post hoc testing using Bonferroni's modification of the *t*-test or non-parametric tests such as the independent-samples Mann–Whitney *U*-test, the Kruskal–Wallis test and Spearman's rho test were applied. Differences with a *p*-value of 0.05 or less (two-sided) were considered significant. The results of the PI, ATP, trypan blue and ^{51}Cr-release assays were normalized to those for the non-irradiated controls.

Results

Effect of single-dose irradiation on the killing capacity of NK cells. The killing capacity of the irradiated NK cells enriched using the methods I-V was measured using the 51Cr-release assay. The results showed that the cytotoxicity of the cells enriched using method I remained highest up to 40 Gy (Figure 1). The parameters of the linear-quadratic model (α and β) based on these data in Figure 1 are presented in Table I. The β values of the cytotoxicity data were positive only with methods II and III, resulting in α/β-ratios of 8.3 Gy and 14.4 Gy, respectively, which are characteristic for acutely responding tissues. The other β values were negative and replaced with 0, and re-fitted, as described above. For these cases, the new α values representing the linear part of the α/β model were added to Table I. The α values were 0.03 Gy^{-1} for non-selected NK populations and 0.07 and 0.09 Gy^{-1} for CD16$^+$ and CD56$^+$ cells, respectively.

The 18 hours post-irradiation AUC values for the cytotoxicity data obtained with the irradiation doses ranging from 0 to 40 Gy (Figure 1) were between 21 and 23 Gy for the non-selected NK cells enriched using methods I-III (Table II), whereas the AUC was approximately 13-14 Gy for CD56$^+$ and CD16$^+$ cells combined.

However, there were no significant differences between the populations (p=0.714).

Effect of single-dose irradiation on the viability of NK cells. For detailed viability studies, irradiated, non-selected NK cells enriched using method II were used. The viability was measured using trypan blue exclusion, PI and ATP measurements. The data are presented in Figure 2. The

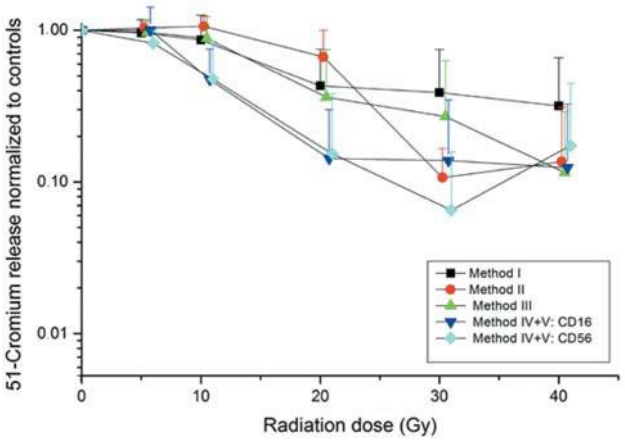

Figure 1. *Natural killer cells enriched using methods I-V were irradiated using doses ranging from 0 to 40 Gy. The cytotoxicity was measured by the ^{51}Cr-release assay 18 hours post-irradiation. The α (Gy^{-1}), β (Gy^{-2}) and the area under the survival curve (Gy) values were determined from the data of these curves. Error bars represent the standard deviation of the mean.*

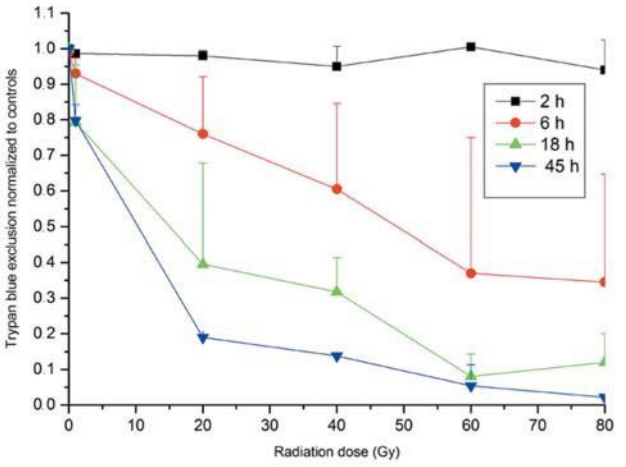

Figure 3. *The long-term effects of single irradiation doses up to 80 Gy on the viability of non-selected natural killer cells were studied by the trypan blue exclusion test 2, 6, 18 and 45 h after irradiation. Error bars represent the standard deviation of the mean.*

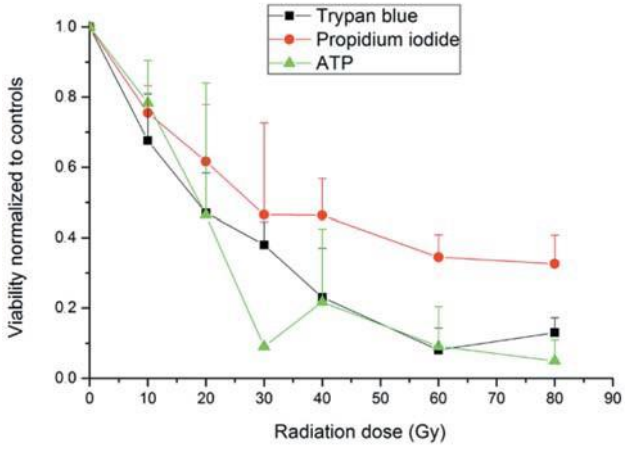

Figure 2. *Viability of non-selected natural killer cells enriched using method II irradiated with a single dose (from 0 to 80 Gy). The viability was measured by trypan blue exclusion, propidium iodide and ATP methods 18 h after irradiation. The results are expressed as fractions of the non-irradiated controls. The α (Gy^{-1}), β (Gy^{-2}) and the area under the survival curve (Gy) values were determined from the data of these curves. Error bars represent the standard deviation of the mean.*

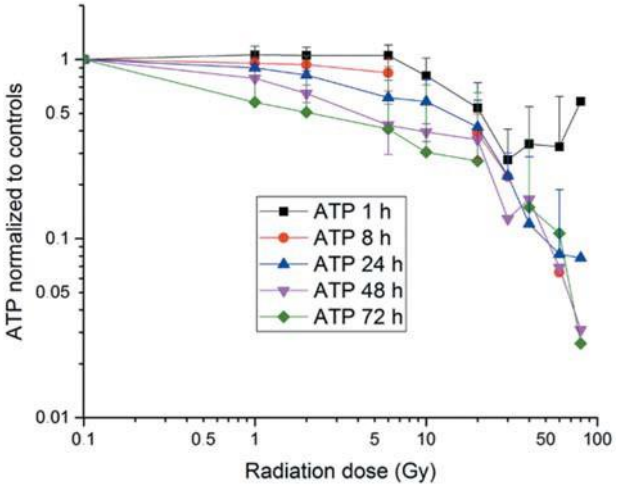

Figure 4. *ATP content of irradiated natural killer cells as a function of irradiation dose and time post-irradiation. Non-selected NK cells were irradiated with single doses ranging from 0 to 80 Gy. The ATP content of cells was measured at time points from 0 to 72 h post-irradiation. Error bars represent the standard deviation of the mean.*

corresponding α and β values are presented in Table III. Only the ATP data resulted in positive β values. The α/β-ratio was 39.6 Gy, corresponding to the acutely responding tissues. For trypan blue exclusion and PI data, the refitted α values were 0.040 Gy^{-1} and 0.019 Gy^{-1}, respectively.

The AUC values obtained with the trypan blue exclusion, PI and ATP methods were 29.9 Gy, 25.6 Gy and 16.8 Gy, respectively. All of these methods were equally good to describe NK cell death caused by irradiation (p=0.387).

The proportion of dead NK cells was evaluated up to 45 h post-irradiation using the trypan blue exclusion method (Figure 3). Two hours after irradiation, practically no effect was observed on viability within the dose range from 0 to 80 Gy.

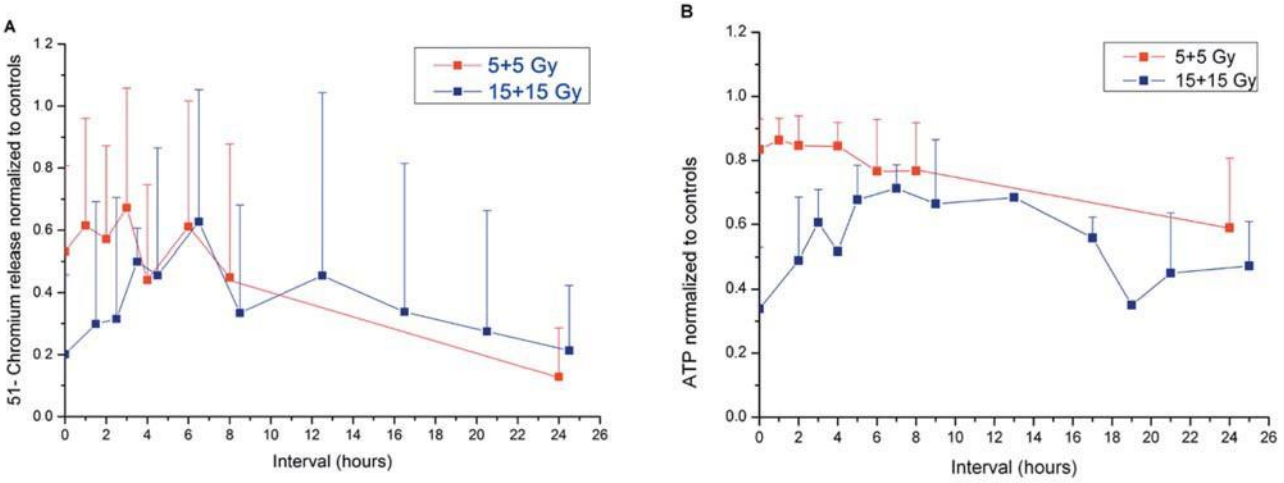

Figure 5. *Natural killer cells enriched using method II were irradiated with total doses of 10 and 30 Gy split into two fractions. The interval between fractions ranged from 0 to 24 h. The radiation effect was measured with ^{51}Cr release (A) and ATP (B). Error bars represent the standard deviation of the mean.*

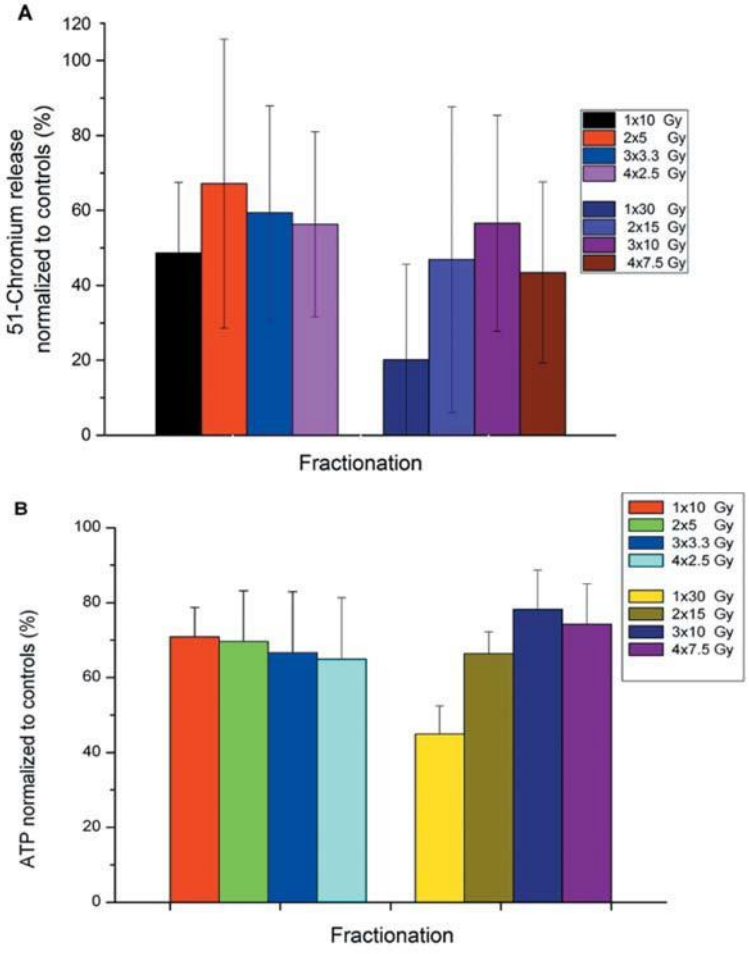

Figure 6. *Natural killer cells enriched using method II were irradiated with doses of 10 Gy and 30 Gy. The target-effector ratio was 1:12.5. The doses were split into several fractions applied at 3-h intervals. ^{51}Cr-release (A) and ATP (B) and were measured and normalized to those of the controls. Error bars represent the standard deviation of the mean.*

Table III. *The linear-quadratic model applied to the survival data of irradiated natural killer cells enriched using method II. Viability was measured using trypan blue exclusion, propidium iodide and ATP methods 18 h after irradiation. The standard errors of the parameters (SE) and p-values are given.*

	Isolation method	α (Gy^{-1})	SE	B (Gy^{-2})	SE	*p*-Value
Trypan blue	II	0.0478	0.011	−2.71546E-4	0.0003	0.000
Trypan blue*		0.0397	0.004	0	0	1
Propidium iodide	II	0.0287	0.002	−1.8565E-4	0.000	0.000
Propidium iodide*		0.0187	0.0018	0	0	1
ATP	II	0.0109	0.006	2.7524E-4	0.000	0.000

*Negative β values were replaced by zeros, and the linear-quadratic model was refitted.

Thereafter, the viability decreased almost linearly up to the dose of 60 Gy, followed by a plateau up to 80 Gy. Consequently, all values in the radiobiological models were strongly dependent on the selected time point after irradiation. The decrease in viability as a function of time is also reflected by the AUC for the trypan blue exclusion data. After 2, 6, 8 and 45 h, the AUC values were 39.5 Gy, 33.5 Gy, 20.4 Gy and 12.5 Gy, respectively. Conversely, the AUC values for living NK cells changed from 69.5 Gy to 10.6 Gy in 48 h.

The ATP content of non-selected NK cells was measured up to 72 h after irradiation using doses from 0 Gy to 80 Gy. The dose range of 0-1 Gy produced practically no changes in the ATP content 2 h after irradiation (Figure 4). From 1 Gy to 6-8 Gy, an elevation of ATP levels was seen (108.9±6.5%). This effect disappeared within 4-6 h. A steep fall was observed with doses between 10 and 40 Gy. Thereafter, a plateau was reached up to doses of 60 Gy and over. During the time course, the ATP levels dropped very rapidly within 1-2 h, followed by a slow and continuous fall for up to 48 h, after which there was hardly any further decrease.

Effects of fractionated irradiation at different intervals on the cytotoxicity and viability of NK cells. When splitting the total dose into two equal fractions applied at different intervals (1, 2, 3, 4, 8, 12, 16, 20 and 24 hours), ^{51}Cr-release measurements at 10 Gy resulted in non-significant changes (Figure 5A). At 30 Gy, a continuous rise of ^{51}Cr release was observed up to 6 h. However, the rise was not significant (p=0.664). A slow decline in values was observed over longer intervals.

The fractionation did not affect the ATP levels of NK cells significantly at the 10-Gy dose (p=0.063). However, at 30 Gy, changing the fraction interval from 0 to 6 hours caused a progressive 2.1-fold rise (p=0.002). After 12 h, a slow decrease was observed until 24 h (Figure 5B).

Effects of fractionated irradiation with different numbers of fractions on the cytotoxicity and viability of NK cells. The total dose of 10 Gy was divided into fractions of 2×5 Gy, 3×3.3 Gy, 4×2.5 Gy applied at 3-h intervals. The cytotoxicity of NK cells was significantly elevated when 10 Gy was divided into two fractions (p=0.04). Splitting 30 Gy into several (up to three) fractions resulted in a 2.7-fold rise in ^{51}Cr release (Figure 6A). However, the increase was not significant (p=0.130). The radiation effects were still detectable after 3 days, although to a lesser extent.

When the total dose of 10 Gy was divided into fractions of 2×5 Gy, 3×3.3 Gy, 4×2.5 Gy applied at 3-h intervals, ATP levels were not significantly affected. However, when 30 Gy was divided into multiple fractions (2×15 Gy, 3×10 Gy, 4×7.5 Gy), a steady rise in the ATP levels was observed up to 1.4-fold that of the controls at 3 fractions (p=0.001) (Figure 6B).

Discussion

The aim of this work was to characterize the radiosensitivity of non-selected NK cells, and $CD56^+$ and $CD16^+$ sub-groups using viability and cytotoxicity as end-points. For viability, we used several methods, including trypan blue exclusion, PI and ATP methods. These methods describe different aspects of cell death. According to our results, they describe NK cell viability and death equally well. Our cytotoxicity and viability data were strongly dependent on the irradiation dose, time after irradiation and NK cell-enrichment methods. We applied mathematical models developed for clonogenic dividing cells to describe the radiation dose–survival relationship to functional data such as cytotoxicity and viability. NK cells do not divide without biological response modifiers (26). The α/β ratio is currently used in practical radiotherapy to calculate radiobiological effects, particularly for different fractionation schemes. It is also useful in describing functional data (27, 28). In our experiments, the α/β ratio of cytotoxicity data measured by the ^{51}Cr release assay was relatively high and of the same order as for acutely responding tissues. The same is true for the viability data obtained by the ATP, PI and trypan blue exclusion methods. The mean inactivation dose $\bar{D}$ is also applicable to non-dividing cell populations and produces more consistent results than other parameters (such as α/β) when comparing

repeated experiments and different cell lines. It quantifies the radiation reactions well over the whole dose range (16). It is recommended by the International Commission on Radiation Units & Measurements to characterize the radiosensitivity of mammalian cells (29). The cytotoxicity AUC values of NK cells enriched by methods I-V were identical even if the cells were non-selected or highly purified.

In some instances, fractionated irradiation enhanced the viability measured by the ATP method and the killing capacity of NK cells *in vitro*. In clinical situations, where daily fractions from 1.8 to 4 Gy are typically used, these results may have some clinical importance.

ATP measurements are used in radiation biology to reflect the radiation damage to ATP synthesis (30). In our pilot work, we measured the intracellular energy production by determining the intracellular ATP, ADP and AMP levels of NK cells by high-performance liquid chromatography. The results indicated that ATP production is stimulated by low-dose irradiation and inhibited by doses higher than 10 Gy; concomitantly, ATP turnover rises. These findings support a hypothesis of mitochondrial excitation at lower radiation dose levels followed by damage at higher doses. The anti-oxidative capacity of the cells seems to be exhausted, and the elimination of radiation-induced radicals seems to be inhibited. Thus, intracellular ATP may reflect the ability of irradiated NK cells to survive.

There are very few radiobiological characteristics of NK cells described in the literature that are comparable with our data. Many reports on the radiobiological characteristics of B- and T-lymphocytes have been published but the comparison of these data is limited because radiobiological data of this type are clearly dependent on factors such as the enrichment and assay methods used, time points of measurements after irradiation and cell cycle (31, 32). In our pilot study, $CD3^+$ T-cells were more radioresistant than $CD16^+$ NK cells, as defined by AUC ($\bar{D}$) values. Our data confirm the findings of Rana *et al.* (13).

In modern radiotherapy, the daily dose fraction most commonly is 2 Gy, delivered at a dose rate ranging from 4 to 6 Gy/min. Even single doses of approximately 10 Gy have been used (33). According to our results, this dosage should cause only little instantaneous reduction of the NK cell cytotoxicity. However, the long-term influence of radiotherapy on NK cells also depends on the total dose, fractionation scheme used and location of the radiation fields (34). *In vivo*, several factors, including biological response modifiers such as interferons and interleukins, alter the radiosensitivity of different NK cell properties (35).

Conclusion

The linear-quadratic model and mean inactivation dose as the AUC were applied to viability and cytotoxicity data of different non-selected and highly purified $CD56^+$ and $CD16^+$ human NK cell populations. The viability and cytotoxicity data were typical for acutely responding human tissues. The AUC method was very useful when comparing different NK populations. When using the linear-quadratic model, the quadratic component β was sometimes negative, and only the linear component α was useful. No *in vitro* post-irradiation recovery of viability or cytotoxicity was detected.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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Action of recombinant interferons and interleukin 2 in modulating radiation effects on viability and cytotoxicity of large granular lymphocytes

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Abstract. We have evaluated *in vitro* the modulating effect of interferon (IFN) α, β and γ as well as interleukin 2 (IL-2) on the radiosensitivity of large granular lymphocytes (LGL) having natural killer cell activity. LGL were treated with IFNs or IL-2 in concentrations from 1 to 1000 U/ml before or after a single or a split dose of irradiation. The viability of LGL was measured by intracellular ATP, and cytotoxicity by a ^{51}Cr release assay. Both viability and cytotoxicity were clearly higher when IFNs and IL-2 were used before irradiation. Some IFNs were slightly radiosensitizing in ATP studies. Only IFN γ in a concentration of 1000 U/ml was significantly radioprotective in cytotoxicity tests when used before irradiation. IL-2 had a significant concentration-dependent radioprotective effect in cytotoxicity when used before or after irradiation, and in the viability of preincubated LGL. No combination of IFNs and IL-2 was more radioprotective than IL-2 used alone. IL-2 retarded the time dependent decrease of ATP and ^{51}Cr release levels after irradiation. According to our results, IL-2 is a radioprotective substance for LGL.

1. Introduction

Natural killer (NK) cells are a group of lymphocytes that are functionally able to lyse target cells without presensitization and restriction by major histocompatibility antigens. Morphologically about 80% of them are the so called large granular lymphocytes (LGL) (Timonen *et al.* 1981). Phenotypically they are characterized by several surface antigens, the best being CD56 + CD3 −. They can kill cancer cells under various conditions (Pross and Baines 1976). For details and other NK cell properties see the review by Robertson and Ritz (1990).

Radiotherapy is an important cancer treatment modality. However, radiation damages the viability, cytotoxicity, and reproductive integrity of NK cells *in vivo* and *in vitro* (Broval and Schacter 1981, Rotstein 1983, Aaran *et al.* 1988), mainly in the post-binding stage of a NK cell cytotoxic event (Zarcone *et al.* 1989, Rana *et al.* 1990).

Biological response modifiers may influence tumours in several ways. Interferons (IFNs) act as oncogene regulators (Samid *et al.* 1987), immunomodulators (Gresser *et al.* 1972) and cytotoxic agents (Gutterman *et al.* 1980). Interleukin 2 (IL-2) activates cytotoxic T and LGL. Lymphokine activated killer cells (LAK) can lyse tumour cells (Grimm *et al.* 1982), and they are used therapeutically (Rosenberg 1984). Reports concerning the effect of IFNs on irradiated NK cells have been conflicting. According to some studies IFNs increase NK activity (Rotstein *et al.* 1983) whereas other studies show that IFN α and γ are ineffective in this respect (Hietanen *et al.* 1990). We (Hietanen *et al.* 1990) and others (Schacter *et al.* 1982, Zarcone *et al.* 1989) have demonstrated that IL-2 can reduce radiation effects on NK cells. However, the information concerning IFNs and IL-2 in combination with ionizing radiation is still sparse and partly conflicting. Therefore, we treated LGL *in vitro*, representing here NK cells, systematically with varying concentrations and combinations of recombinant IFN α, β γ and IL-2 before or after a single or split dose of γ-radiation. As end points, viability by intracellular ATP and the killing capacity of LGL assessed by ^{51}Cr release were used. In order to be able to compare the effects of IFNs and IL-2 on irradiated LGL, radiobiological parameters D_0, n, α, β and the mean inactivation dose $\bar{D}$ are presented.

2. Methods

2.1. *Culture conditions*

Cells were cultured in RPMI 1640 medium (Orion Diagnostica, Finland) with 10% heat-inactivated foetal calf serum (Flow Laboratories, UK), L(+)glutamine 0·3 g/l (Fluka, Switzerland) and gentamycine 20 μ/ml

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(Flow Laboratories) at 37°C in a humidified air atmosphere with 5% CO_2.

2.2. *Enrichment of LGL*

LGLs were purified from fresh buffy coats obtained from the Finnish Red Cross Transfusion laboratory using the modified method of Timonen and Saksela (1980), which was described previously (Hietanen *et al.* 1990). The permission to use buffy coats for research purposes was obtained from the ethical committee of the Finnish Red Cross.

In short, mononuclear cells were isolated using a Ficoll–Paque gradient. The lymphocyte fraction was run through a Nylon wool column. Non-adherent cells were centrifuged in two discontinuous Percoll gradients. Of the resulting cells, 85·2 ± 9·1% (1 SD) were LGLs. The phenotypic analysis of isolated cells has been published elsewhere (Hietanen *et al.* 1993). The proportion of macrophages and B cells was 1 ± 1%. After purification, LGLs were stored over night at 4°C and used the following day with a 99 ± 0·5% viability in these experiments.

2.3. *IFN and IL-2 treatment of LGL*

We used recombinant IFN α2b (Schering Corp., CA, USA), recombinant IFN β (Kyova Hakko Kogyo Co., Ltd, Japan), recombinant IFN γ (Genenteck, CA, USA), and recombinant IL-2 (Janssen Biochimica, Belgium) in concentrations ranging from 1 to 1000 U/ml with single doses of irradiation and in the recovery experiments before or after irradiation. We applied them only before irradiation in concentrations of 100 U/ml for tests where we combined IL-2 with IFN α, β or γ; and for calculations of radiobiological parameters; as well as IL-2 in the split-irradiation dose experiments in concentrations from 10 to 1000 U/ml.

LGLs were incubated in U-bottomed, 10-ml plastic tubes at a concentration of $1{\cdot}25 \times 10^6$ cells/0·9 ml medium added with 0·1 ml IFNs or IL-2. Controls were treated in the same way but instead of IFNs or IL-2, 0·1 ml 0·9% NaCl was added to the tubes. The incubation time was 72 h, before or after irradiation. According to preliminary studies, this time period was long enough for IFNs and IL-2 to influence the ATP content and the cytotoxicity of the LGL without cell division.

2.4. *Irradiation of LGL*

For the irradiations a ^{137}Cs device (Gammacell 2000, Mølsgaard Medical, Denmark) was used. The dose-rate was 4·3 Gy/min. Single doses of 30 Gy were given or two doses of 15 + 15 Gy with an interval of 4 h. These conditions were shown to be optimal in our earlier study (Hietanen *et al.* 1993). For the dose–response curves, single varying radiation doses were used. Irradiation of well-oxygenated LGL was carried out at room temperature.

2.5. *Controls*

Three types of controls were employed; A, unirradiated and without either IFNs or IL-2-treated LGL; B, only irradiated LGL for experiments where incubation with IFNs and IL-2 was done before irradiation; and C, corresponding irradiated controls for experiments with postirradiation incubation.

2.6. *ATP measurement*

The viability of LGL was measured using a bioluminescence method for intracellular ATP using commercial kits from LKB Wallac (Finland). This method is described elsewhere (Kangas *et al.* 1984, Hietanen *et al.* 1990). Triplicates were used and results were calculated as fractions of values of the cells in control A.

ATP measurements were performed 6 h after irradiation in experiments where LGLs were preincubated with IFNs or IL-2, and immediately after the incubation time in experiments where the LGLs were irradiated before incubation with these drugs. In split-dose studies, ATP determinations were performed 6 h after the last radiation dose. In recovery experiments we followed ATP levels for 5 days.

2.7. *^{51}Cr release assay*

The killing capacity of LGLs was monitored by a modified ^{51}Cr release assay developed by Timonen *et al.* (1981). This method has been described earlier (Hietanen *et al.* 1990). In brief, K-562 cells served as target cells. An effector/target ratio of 12·5:1 was used. All experiments were done as triplicates and the mean calculated. For calculating relative cytotoxicity the following formula was used:

$$Cx(\%) = ((exp - spont)/(max - spont)),$$

where *exp* is experimental release, *spont* is spontaneous release, and *max* is maximal release from K-562 cells lysed with a 1% Triton X-100. The spontaneous release never exceeded 10% of the maximal release. The results are reported as fractions in cytotoxicity of control A cells. ^{51}Cr release assays were performed at the same points in time as reported for the ATP measurements.

2.8. *Models for radiation dose–response curves*

For the radiation dose–response curves we used (1) the single-hit, multitarget-model (Puck and Marcus 1956), (2) the linear–quadratic model (Kellerer and Rossi 1972), and (3) the mean inactivation dose model (Kellerer and Hug 1972):

(1) single-hit, multitarget model:

$$f = c[1 - (1 - e^{-D/D_0})^n]$$

(2) linear–quadratic model:

$$f = c[e^{-(\alpha D + \beta D^2)}]$$

In formulae 1 and 2, c represents the starting point of the curve on the y-axis with a dose of 0 Gy.

(3) $\bar{D} = \int_0^\infty S(D)d(D)$,

where f is surviving fraction, D is radiation dose, D_0 is dose that decreases the number of viable cells to e^{-1} of its original value, and n is the extrapolation number. The linear–quadratic model has two inactivation parameters, the linear term α and the quadratic term β. The mean inactivation dose was calculated by first fitting a linear–quadratic model to the experimental data, then reconstructing the best-fit curve and obtaining the area under this curve (*AUC*) by integrating the dose from 0 Gy to ∞. *AUC* corresponds to the mean inactivation dose.

2.9. *Curve fitting*

Curves were fitted to the experimental data using the Marquardt algorithm in the P. Fit program, version 5.1 (Fig. P Software Corp., Durham, NC, USA) by the iterative, weighted least-squares method.

2.10. *Statistical analysis*

All measurements were done as triplicates. Every single value in the results represents the mean of 4–6 independent tests from different donors. The statistical analyses were calculated by the BMDP statistical program (BMDP Statistical Software, Inc., Los Angeles, CA, USA) using *t*-tests for matched samples or paired variables (program 3D), one-way analysis of variance (7D), and one-way analysis of covariance (1V) to discover statistically significant differences between the effects of different concentrations of IFNs and IL-2 on LGL irradiated with single, or fractionated doses, or on the radiobiological parameters of LGL.

3. Results

3.1. *IFN α, β, γ and IL-2 combined with a single dose of irradiation*

In the first phase of this study we treated LGL with varying concentrations of IFN α, β, and γ as well as with IL-2 before or after irradiation. The results for viability are shown in Figure 1a and for cytotoxicity in Figure 1b. In general, the absolute levels of ATP and ^{51}Cr release were much higher when IFNs and IL-2 were used before irradiation than after it. However, when the levels were compared with those of corresponding irradiated control LGL (controls B and C), they appeared to be in the same range.

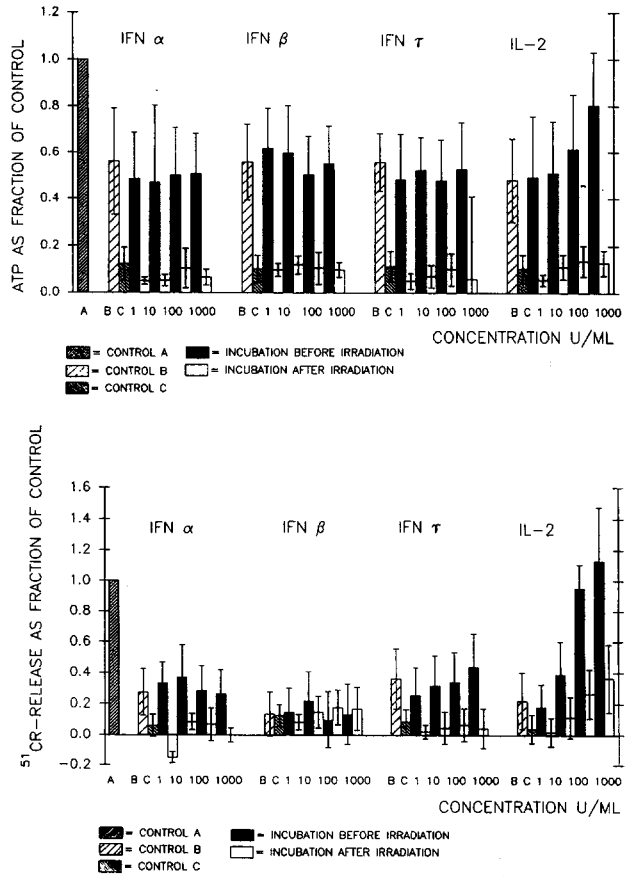

Figure 1 a and b. Effect of different concentrations of IFN α, β, γ and IL-2 viability and cytotoxicity of irradiated (30 Gy) LGL when given 72 h before or after irradiation. Viability was measured by ATP (a) and cytotoxicity by ^{51}Cr-release method (b). Controls used were: A, unirradiated, neither with IFN's nor IL-2 treated LGL; B, only irradiated LGL controls for experiments having incubation with IFN's or IL-2 before irradiation; and C, only irradiated controls for LGL having postirradiation treatment with IFN's or IL-2. Results are expressed as a fraction of control A. Error bars represents 1 SD of the mean.

3.1.1. *IFNs.* When we used IFN α, β and γ before irradiation, in most cases they decreased ATP levels 8–16% in comparison with irradiated controls (control B) and as much as 60% when they were used after it (control C). Thus, they were sensitizing LGL cell to radiation. However, these effects were not statistically significant. There was no concentration–response relationship detectable. In cytotoxicity studies, IFN α and β in some concentrations showed insignificant elevations of cytotoxicity above control levels, when used prior to irradiation. IFN γ demonstrated to some extent a concentration–response relationship if used before irradiation. A concentration of 1000 U/ml elevated cytotoxicity to 1·26 times above the levels of the irradiated controls (control B) ($p = 0{\cdot}008$). When we used IFN α, β and γ after irradiation, they caused no statistically significant changes in cytotoxicity.

3.1.2. *IL-2.* In contrast with IFNs, IL-2 demonstrated a clear concentration–response relationship. When added before irradiation, 10 U/ml IL-2 elevated ATP levels above those in the irradiated control (control B) and significantly at concentrations > 100 U/ml. The latter concentration elevated ATP levels 1·3-fold ($p = 0{\cdot}02$) and 1000 U/ml up to 1·7-fold ($p = 0{\cdot}02$). In some single tests ATP levels exceeded also those in unirradiated controls (control A). Application of IL-2 after irradiation did not significantly elevate ATP levels.

In cytotoxicity tests IL-2 showed an even stronger concentration–response relationship than in the ATP experiments. Preincubation with 100 U/ml caused a 4·3-fold ($p = 0{\cdot}0007$) and 1000 U/ml a 6·9-fold ($p = 0{\cdot}003$) difference in cytotoxicity compared with the corresponding control (control B). The latter concentration increased the cytotoxicity 1·13-fold over that in the unirradiated control (control A). If used after irradiation, the above-mentioned concentrations elevated ^{51}Cr release 6·9 ($p = 0{\cdot}01$) and 9·5-fold ($p = 0{\cdot}01$) in comparison with the respective control (control C).

3.2. *Effect of IL-2 on LGL irradiated with a single dose of 30 Gy, or with two equal doses of 15 Gy each*

In previous studies it was shown that division of a radiation dose into fractions diminished damage in LGL (Hietanen *et al.* 1989, Hietanen and Pitkänen 1992). We evaluated the possibility of protecting LGL from additional radiation damage by combining split-dose radiation and IL-2. We measured viability and cytotoxicity of LGL pretreated with different IL-2 concentrations and irradiated them either with a single dose (30 Gy) or with two fractions (15 + 15 Gy). The difference between the curves describing single and split-dose irradiation effects rose in viability 1·9 times and in cytotoxicity studies 2·9 times when the IL-2 concentration was increased from 10 to 1000 U/ml. However, the slopes of both pairs of curves did not differ significantly. Thus, in addition to IL-2, use of split-dose irradiation gave no additional protection against radiation damage in LGL.

3.3. *Effect of combinations of IFNs and IL-2 on LGL before a single radiation dose*

IL-2 proved to be effective in abolishing radiation damage in LGL. In some cases IFNs were also radioprotective. We studied whether or not it would be possible to protect LGL more by combining IL-2 with IFNs. The results are shown in Figure 2a and b. They are expressed as fractions of control A. In both viability and cytotoxicity studies no combination gave significantly better results than IL-2 used alone.

3.4. *Recovery of LGL as a function of the time after irradiation, with or without IFNs and IL-2*

We treated LGL in varying concentrations of IFN α, β and γ as well as IL-2 before or after irradiation (30 Gy) and followed both viability and cytotoxicity up to 5 days. There was no recovery in the intracellular ATP content or cytotoxicity of IFN-treated LGL during the observation time. On the contrary, they decreased as a function of time. However, high concentrations of IL-2 were capable of slowing down the decline in the viability and cytotoxicity, and inducing in single cases of the cytotoxicity measurements some degree of recovery.

3.5. *Radiobiological characteristics of IL-2 treated and irradiated LGL*

We used single-hit multitarget, linear–dquadratic and mean inactivation dose models in determining the radiobiological characteristics of LGL, which were incubated with IFN α, β, γ and IL-2 (100 U/ml) before single-dose irradiation using different doses. D_0, n, α, β and $\bar{D}$'s, which are clearly dependent on the concentrations of the agents mentioned above, are presented in Table 1. IL-2 broadened the 'shoulder' and elevated the level of the curve of the radiation–dose–response plot in the viability and cytotoxicity experiments. Therefore, its radioprotective effect on LGL was best reflected by $\bar{D}$ and n's. In the ATP studies, $\bar{D}$'s for IL-2 treated LGL were significantly higher ($p \leq 0{\cdot}05$) than those for the controls, or with IFN α or β, but not with IFN γ-incubated cells. Also in the cytotoxicity studies, $\bar{D}$'s for IL-2 treated LGL were higher than $\bar{D}$'s ($p \leq 0{\cdot}05$) for all

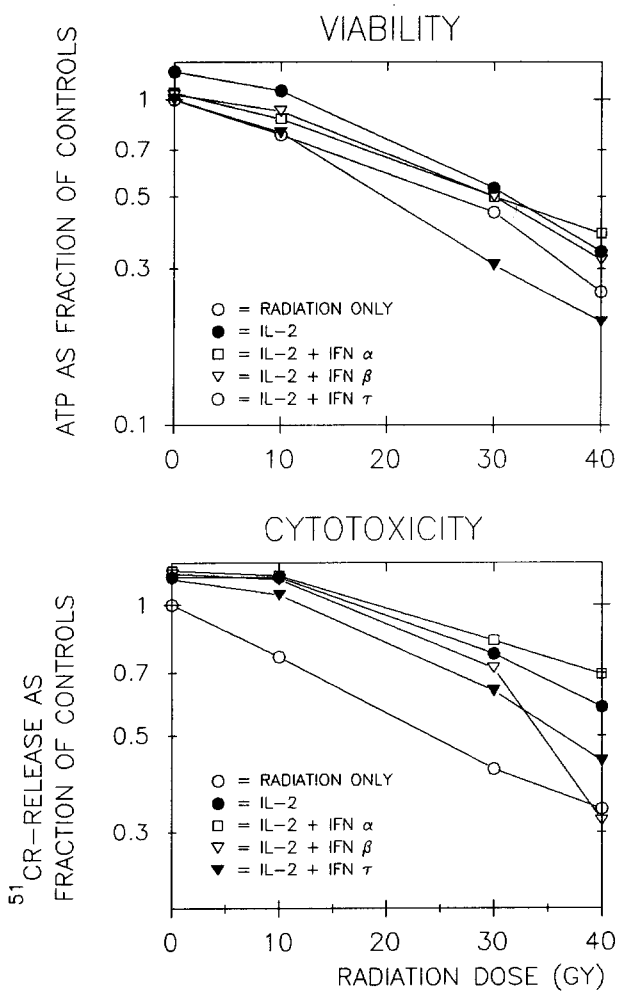

Figure 2 a and b. Viability (a) and cytotoxicity (b) of LGL, which were pretreated for 72 h with 100 U/ml IL-2 in combination with 100 U/ml IFN α, β or γ and then irradiated with doses ranging from 0 to 40 Gy. Results are calculated as fractions or unirradiated LGL controls, which were treated neither with IFN's nor IL-2 (control a).

IFN-treated cells. The *n* of the curve of the ^{51}Cr release but not of the ATP data for IL-2 was significantly higher ($p \leq 0{\cdot}05$) than levels for control cells or IFN-treated cells.

4. Discussion

We have studied the hypothesis that recombinant IFNs and IL-2 can modulate radiation effects on LGL. We could demonstrate that IL-2 can reduce radiation effects whereas IFNs were clearly less effective in this respect. In fact, IFNs were slightly radiosensitizing on some occasions. Our IL-2 results corresponded with those of Schacter *et al.* (1982). They were in disagreement with some earlier IFN results. For example, Rotstein *et al.* (1983) showed that IFNs were radioprotective in cells with NK activity. In our study only recombinant IFN γ in high concentrations was found to increase cytotoxicity but not the ATP content of irradiated LGL in a dose-dependent fashion, and only when used before irradiation. The reason for the discrepancy may be that Rotstein *et al.* used only Ficoll–Isopaque-purified lymphocytes as NK cells and partially purified IFN-α(PIF), both preparations which are rather impure. In addition, Ortaldo *et al.* (1983) reported considerable differences between various IFN species and concentrations in modulating immunological functions. We used recombinant IFNs and IL-2 as well as Percoll-isolated LGL containing *c.* 85% LGL, predominantly CD56 + and CD16 + cells (Hietanen *et al.* 1993).

Table 1. Radiobiological characteristics of irradiated LGL with IFN α, β, γ and IL-2 (100 U/ml) treatment (± 1 SD)

	D_0(Gy) ± SD	*n* ± SD	α(Gy^{-1}) ± SD	β(Gy^{-2}) ± SD	*D*(Gy) ± SD
ATP					
Control	20·3 ± 4·5	2·0 ± 0·4	0·020 ± 0·006	0·0004 ± 0·0003	30·6 ± 9·3
IFN α	20·5 ± 5·7	2·2 ± 0·3	0·020 ± 0·009	0·00050 ± 0·00009	26·6 ± 5·2
IFN β	16·9 ± 1·9	1·7 ± 0d1	0·010 ± 0·007	0·0010 ± 0·0002	23·1 ± 1·8
IFN γ	27·9 ± 15d9	1·6 ± 07	0·01 ± 001	0·0005 ± 0·0004	32·3 ± 12·9
IL-2	18·1 ± 3·1	3·7 ± 2·9	0·003 ± 0·002	0·0005 ± 0·0006	39·5 ± 8·8
^{51}Cr-release					
Control	25·8 ± 11·8	1·7 ± 0·3	0·0100 ± 0·0004	0·00020 ± 0·00008	31·8 ± 14·3
IFN α	19·8 ± 9·4	2·1 ± 0·6	0·03 ± 0·01	0·0002 ± 0·0003	32·2 ± 13·9
IFN β	13·9 ± 6·4	3·3 ± 1·7	0·020 ± 0·002	0·0009 ± 0·0001	27·2 ± 15·9
IFN γ	24·1 ± 11·2	1·7 ± 0·7	0·03 ± 0·03	0·0003 ± 0·0001	31·7 ± 17·2
IL-2	24·3 ± 11·6	4·0 ± 0·7	0·0090 ± 0·0003	0·0002 ± 0·0003	51·4 ± 13·3

The stimulation of LGL by IFNs and IL-2 is dependent on incubation time and concentration. We used a long incubation time (72 h) and a broad range of concentrations to assure an observable effect, if any, on LGL. Bandyopadhyay *et al.* (1987) demonstrated that IFN α and β induced maximal NK cell-mediated killing after 4 h of incubation, while IFN γ and IL-2 required 18 h. Very long incubation times with IL-2 induced proliferation of LGL (Timonen *et al.* 1982). However, after only 3 days we could detect no increase in the number of LGL by extensive counting (using a Bürger chamber). This has been confirmed by others

(T. Timonen, personal communication). Therefore, the increase in the viability and cytotoxicity was not caused by an increased number of LGL. James *et al.* (1983) and Gerber *et al.* (1989) have shown for T lymphocytes that IL-2 activates the cells and initiates DNA synthesis and makes T cells more radioresistant. So, IL-2 is radioprotective for LGL cells, as for all cells with an IL-2 receptor, possibly through the IL-2-induced DNA synthesis.

The concentration range of the IFNs and IL-2 we used, cover the levels measured in the plasma of cancer patients treated with those substances. Steineck *et al.* (1990) observed IFN α-2A plasma concentrations mainly between 0 and 1000 U/ml in renal cell carcinoma patients. Rinehart *et al.* (1986) had IFN-β serum levels of several hundred U/ml and IFN γ up to 1500 U/ml in renal cell cancer patients treated with these substances. Konrad *et al.* (1990) studied pharmacokinetics of human recombinant IL-2 and observed plasma concentrations, typically 650 U/ml for a dose of 10^6 units/m^2. We have used IL-2 in our institution at $2{\cdot}4$–$4{\cdot}8 \times 10^6/m^2$ subcutaneously (s.c.) in renal cell carcinoma patients and measured plasma concentrations up to 100 U/ml (unpublished data).

We measured not the survival for example by s.c. clonogenic cells but the viability of LGL by a less common method, the measurement of intracellular ATP. Radiation damages DNA, which changes purine nucleotide metabolism leading to increased ATP consumption and eventually to depletion. These events may lead to cell death by a cellular 'suicide response' (Altman *et al.* 1970, Cohen *et al.* 1985, Cohen and Barankiewicz 1987). Consequently, the ATP measurement used by us is a convenient method to estimate cell viability and interphase death. It is already used in cytostatic (Kangas *et al.* 1984) and radiation studies (Aaran *et al.* 1988).

Intracellular ATP and ^{51}Cr release levels were definitively higher when LGL were preincubated with IFNs or IL-2 before irradiation in comparison with a postirradiation situation. There may be several reasons for this phenomenon. First, as shown in this and in our earlier paper (Hietanen *et al.* 1993), the ATP content and cytotoxicity of irradiated LGL decrease with time. In this study, ATP and ^{51}Cr release assays were done 6 h after irradiation, when LGL were preincubated with IFN and IL-2, and 72 h after irradiation when postirradiation incubation was applied. So, in the latter case, the parameters had *c.* 3 days longer to decrease. Second, IFNs and IL-2 induce the maturation of LGL (Saksela *et al.* 1979). Mature cells are more radioresistant than the corresponding blasts. For example, mature T cells are known to be more radioresistant than pre-T and resting T cells (Merluzzi 1984). In addition, IL-2 increases the radioresistance of T lymphocytes by inducing DNA synthesis (James 1983, Gerber 1989). This may be true also for the LGL.

Combinations of IL-2 with IFNs did not only enhance but in some cases even reduced, the radioresistance of LGL. Similar phenomenon has been described also by others. Sone *et al.* (1988) found that addition of exogenous recombinant IFN α and β resulted in significant inhibition of LAK activity against the Daudi cell line. Contradictory results have also been published by Silagi *et al.* (1988) where an additive effect was observed when using recombinant murine IFN γ and recombinant human IL-2 against B16 melanoma.

The radiobiological parameters presented in this study were intended to reflect more relations between the effects of IFN α, β, γ or IL-2 on irradiated LGL than to be regarded as absolute values. The $\bar{D}_0$, n, α, β and $\bar{D}$'s obtained here for unirradiated, untreated controls (control A) were different from those in our earlier study (Hietanen *et al.* 1993). In the present study, LGL were incubated 72 h before irradiation whereas in the earlier study no incubation was used. It was necessary to add a correction coefficient c to the single-hit, multitarget and linear–quadratic formulae to mark the starting point of the curve on the y-axis at a dose of 0 Gy because IL-2 elevated both the ATP and cytotoxicity levels of unirradiated control LGL (controls B and C), whereas some IFNs lowered them. The mean inactivation dose model based on the linear–quadratic method (see §2) was most sensitive in describing the differences between the effects of IL-2 and IFNs. It takes into consideration the overall elevation of ATP and ^{51}Cr release curves caused by IL-2, the broadened 'shoulder' and the slope. In the single-hit, multitarget model the n reflects more the 'shoulder' whereas D_0 describes only the slope of the exponential part of the curve.

In conclusion, IL-2 reduces radiation damage expressed by LGL, depending on the IL-2 concentration, incubation in relation to the time of irradiation, the time intervals between irradiation and the observations, and the radiation dose. IFN γ is clearly less effective than IL-2 in this respect and IFNα and β are slightly radiosensitizing agents. This might also have consequences *in vivo* during radiotherapy or in radioactive hazards in boosting NK activity with IL-2. In fact, Cameron *et al.* (1990) demonstrated that IL-2 with or without TIL (tumour infiltrating lymphocytes) in combination with radiotherapy was significantly more effective in treating murine adenocarcinoma metastases than radiation alone.

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Restoring Natural Killer Cell Cytotoxicity After Hyperthermia Alone or Combined with Radiotherapy

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Abstract. *Background: The aim of the present study was to investigate in vitro the effect of hypo- and hyperthermia alone or in combination with irradiation on natural killer cell (NK) cytotoxicity, recovery of this function and the possibility of preventing damage to or enhancing cytotoxicity recovery using interferons (IFNs) α, β, and γ and interleukin-2 (IL-2). Materials and Methods: We used non-selected NK cells and measured their cytotoxicity using the ^{51}Cr release assay. Temperatures ranging from 31-45˚C and thermal treatment times from 0-180 min were assessed. IFNs were applied at concentrations from 0-1,000 IU/ml and IL-2 from 0-450 IU/ml. The range of irradiation dose was from 0-30 Gy. Results: We detected no significant differences in cytotoxicity at temperatures from 31-37˚C. The most significant decrease in cytotoxicity was observed between 41 and 42˚C (p=0.0010), and heating NK cells at 42˚C for 180 min almost completely abolished this function. NK cell cytotoxicity largely recovered during the first 24 h, depending on the heating time. IFN-α, β, and γ demonstrated no concentration-dependent ability to aid in recovery when used before or after the thermal treatment. In contrast, IL-2 restored cytotoxicity in a concentration- and incubation time-dependent manner and was equally active when used before, during or after heating. NK cells were heated at 42˚C for various times and then irradiated with a single dose or first irradiated and then heated; however, no statistically significant differences were observed (p=0.520). An approach of IL-2 treatment followed by radiation and heating was the most effective in restoring NK cytotoxicity (p=0.000). Conclusion: NK cell cytotoxicity is impaired in vitro at 42˚C and above, with possible partial recovery. IL-2, but not IFNs, was able to restore NK cell cytotoxicity in a concentration-dependent manner. IL-2 can also reverse the damage caused by combined hyperthermia and irradiation.*

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In humans, hyperthermia is defined as a temperature greater than 37.5 or 38.3˚C (99.5-100.9˚F), depending on the publication (1, 2). Hyperthermia causes various changes in cellular macromolecules, including unfolding and aggregation of proteins as well as DNA damage (3). It is well-known that hyperthermia kills both normal (4) and tumor cells (5, 6), though certain types of cancer are more sensitive to hyperthermia than normal cells (7). In addition, hyperthermia has an influence on blood flow and on the tumor microenvironment *in vivo* and can act as an adjuvant for the immune system (8).

Indeed, hyperthermia was one of the first therapies used to treat cancer (9), and hyperthermia is currently applied as a systemic treatment to the entire body, local and regional or as ablation treatment (tissue burning) (10). Several approaches can be utilized to induce hyperthermia, including conduction, convection and radiation techniques. Also, bioactive, chemical, mechanical or electromagnetic techniques, with or without nanoparticles, have been used non-invasively, semi-invasively or invasively (10). For cancer therapy, hyperthermia can be applied alone (11) as well as combined with cytokines (12), radiotherapy (13, 14) or chemotherapy (15, 16). However, to date, there exist no reports regarding the *in vivo* combination of hyperthermia, radiotherapy, and cytokines with or without cytostatic drugs.

Temperatures from 38-42˚C are applied for whole-body hyperthermia, whereas 40-45˚C is typically used for loco-regional therapies. Conversely, higher temperatures, ranging from 60-250˚C, are utilized in ablative treatment. The heating times to reach the steady state are from 30 to 180 min in systemic therapy and the entire treatment time from 120 to 720 min. The corresponding times for local/regional treatments vary from 5 to 50 min and from 30 to 90 min, respectively (10).

Natural killer (NK) cells constitute one of the body's non-specific defenses against tumor cells and microbial pathogens (17), and depending on the heating time, *in vivo* NK cell activity and blood levels can be enhanced at temperatures within the fever range (38.3-42˚C) (21-23). However, *in vitro* NK cell viability and cytotoxicity are decreased at higher temperatures (18, 19). NK cell-induced tumor lysis itself is more thermosensitive than the recognition and binding functions of NK cells (20). Further investigations into the roles of major histocompatibility complex (MHC) class I, MHC Class I-like molecule (MICA), heat shock proteins (HPS), NK cell surface natural-killer group 2, member D (NKG2D) and lipid raft clustering (21) have been reported.

Whole-body hyperthermia increases IL-2 and NK cell activity (22, 23). Hypothermia also enhances NK cell activity in healthy individuals at temperatures as low as 35˚C (24, 25), though activity of these cells was reportedly decreased during perioperative hypothermia (26).

In the present study, we systematically examined the effects of hypothermia and hyperthermia (31-45˚C) and heating times on the cytotoxicity of non-selected NK cells *in vitro*, as measured using a ^{51}Cr release assay. In addition, we assessed the effects of IFN-α, β and γ as well as IL-2 in preventing thermal damage to NK cells and their recovery. The combined effects of thermal treatment and radiation on NK cell cytotoxicity and the ability of IL-2 to reverse the damage incurred were also investigated.

Materials and Methods

NK cell enrichment. NK cells were enriched from buffy coat samples obtained from the Finnish Red Cross Transfusion laboratory, with permission of the ethical committee of the Finnish Red Cross Blood Service (customer number 6129, approval number 331/2013, tutkijaluvat@veripalvelu.fi) in accordance with the Finnish law.

We used a method previously described to isolate non-selected NK cells (method II in Hietanen *et al*. 2015) (27). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll-Paque gradient, plastic and nylon wool adherence and a discontinuous Percoll gradient. The yield, purity, viability, cytotoxicity and radiation sensitivity of the enriched NK cells was described in a previous report (27).

Thermal treatment. NK and target K-562 cells were suspended in 1 ml of medium and incubated at different temperatures in a water bath or an incubator in a 5% CO_2 and humified atmosphere for time periods typically ranging from 0-180 min. Preliminary studies showed no differences between the results obtained using a water bath or an incubator. Temperatures were measured using a Fluke 51 K/J thermometer (John Fluke, Everet, WA, USA) with an accuracy of +/–0.1˚C, and pH was controlled with phenyl red present in the medium. Cytotoxicity measurements were performed immediately after the thermal treatment, with the exception of recovery studies, for which they were performed up to 72 h after the thermal treatment.

Irradiation. The enriched NK cell populations were gamma-irradiated, as described earlier (28). In brief, we used a ^{137}Cs device (GAMMACELL 2000, Mølsgaard, Denmark) at a dose rate of 4.1 Gy/min at room temperature (20˚C). Well-oxygenated NK cells were irradiated in U-bottom plastic tubes (BD Falcon, Franklin Lakes, NJ, USA), and the dose was controlled using lithium fluoride thermoluminescence dosimetry. NK cells were irradiated using single doses from 0 to 30 Gy.

IFN α, β γ and IL-2. We used recombinant IFN-α2b (Schering-Plough, Kenilworth, NJ, USA), recombinant IFN-β (Kyova Hakko Kogyo Co, Ltd, Tokyo, Japan), recombinant IFN-γ (Genenteck, San Francisco, CA, USA), and recombinant IL-2 (Janssen Biochimica, Beerse, Belgium), as reported previously (28). In brief, we incubated NK cells with IFN-α, β and γ at concentrations ranging from 1 to 1,000 U/ml for 24 h at 37˚C before or after heat treatment. NK cells were incubated with IL-2 for times ranging from 0-140 h at 1.75 to 450 IU/ml before or after heating at 37˚C and during heating at the indicated temperatures. Samples of 1.25×10^6 NK cells/0.9 ml medium were incubated with 0.1 ml IFNs or various concentrations of IL-2 in U-bottomed, 10 ml plastic tubes. Controls were treated in the same manner, with 0.1 ml of 0.9% NaCl instead of cytokines.

Measurement of NK cell killing capacity. The cytotoxicity of NK cells was measured using a modified ^{51}Cr release assay (29). Briefly, we used a target:effector ratio of 1:12.5. The effector NK cells were thermal treated with or without cytokines and/or irradiation, and measurements were performed in triplicate. Cells in 96-well U-bottomed microtiter plates were incubated for 18 hours at 37˚C in an incubator with a humified atmosphere containing 5% CO_2. The harvested supernatants were measured using a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The relative cytotoxicity was calculated using the following formula: Cx (%)=((exp.-spont.)/(max.-spont.) ×100), where exp. is the average of the experimental wells, spont. is the spontaneous release, and max. is the maximal release from K-562 cells lysed with 1% Triton X-100. The mean values of triplicate samples were used in further calculations. The results were expressed as a percentage of non-heated, non-irradiated and non-cytokine-treated controls.

Cells of an erythroleukemia cell line K-562 (30) were used as the target cells. The cells were grown in RPMI 1640 (Orion Diagnostica, Espoo, Finland) with 10% temperature-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), 0.3 g/l L(+) glutamine (Fluka, Buchs, Switzerland) and 20 mg/ml gentamicin (Flow Laboratories, Irvine, Scotland).

Data presentation and statistical analyses. The results of the ^{51}Cr release assays were expressed as a percentage of the unheated NK cell controls at 37˚C. Every value in this report represents the mean of several independent tests using material from different donors. The results are presented as the means±standard deviation (S.D.). The data were analyzed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). We used analysis of variance (ANOVA) to test the significance of differences between the effects of the different temperatures and thermal treatment times with or without cytokines and irradiation. *Post hoc* analysis was performed using Bonferroni correction of *t*-test results. The constituent ratios were compared using the χ^2 test (Chi-square test) and the Fisher's exact test. The Kruskal-Wallis test and the Mann-Whitney *U*-test were used for data

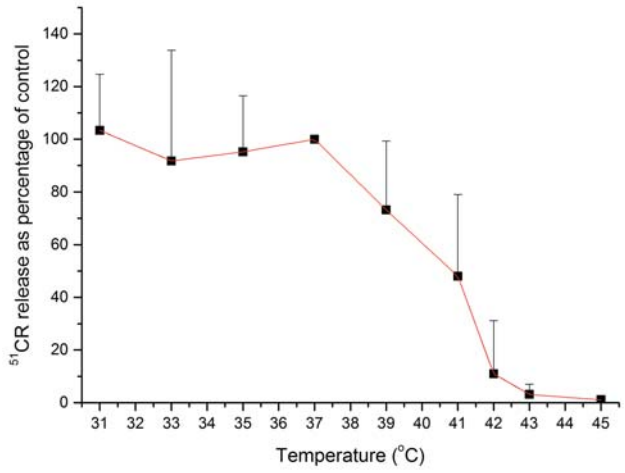

Figure 1. *Cytotoxicity of NK cells at different temperatures. NK cells were incubated for 180 min at temperatures ranging from 31 to 45˚C. The error bars represent the standard deviation of the experiments (n=2-27).*

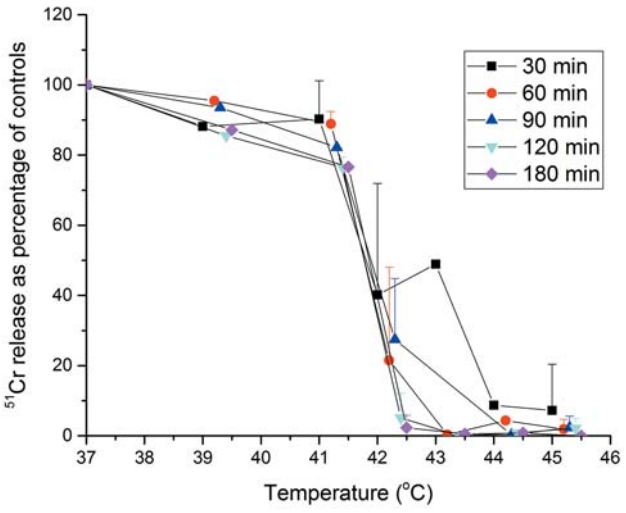

Figure 2. *The effects of different incubation times and temperatures on NK cell cytotoxicity. NK cells were incubated for 30, 60, 90, 120 and 180 min at temperatures ranging from 39 to 45˚C, and cytotoxicity was measured. The error bars represent the standard deviation of the experiments (n=2-27).*

that could not be assessed *via* ANOVA (*e.g.*, data that were not normally distributed). Differences were considered to be significant at $p<0.05$ (two-sided).

Results

Effect of temperature and heating time on NK cell cytotoxicity. The effect of temperatures ranging from 31 to 45˚C for 180 min on NK cell cytotoxicity was investigated in this study. Although there was no change in cytotoxicity from 31 to 37˚C, a continuous decrease in cytotoxicity was observed above this temperature, and cytotoxicity was near zero at 43˚C (Figure 1).

In ensuing experiments, temperatures ranging from 39 to 42˚C were studied in more detail. NK cells were incubated for 30, 60, 90, 120 and 180 min at these temperatures, and cytotoxicity was measured. Temperature elevation from 39˚C to 41˚C did not significantly affect ^{51}Cr release levels ($p=0.318$) at any heating time, whereas an increase from 41˚C to 42˚C resulted in a clear reduction of ^{51}Cr release ($p=0.010$) at all heating times (Figure 2).

To explore the duration of hyperthermic treatment necessary for an effect on the cytotoxicity of non-selected NK cells, cells were incubated at 42˚C for various times, ranging from 0 to 180 min. We found that cytotoxicity decreased almost linearly at a logarithmic scale, reaching almost 0% of the cytotoxicity of control cells after 180 min (Figure 3).

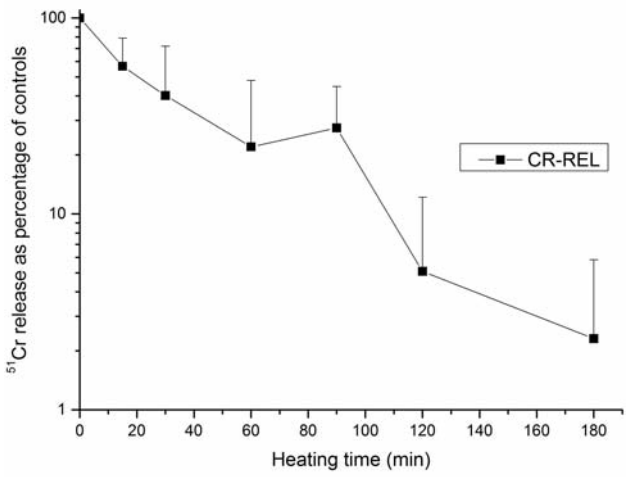

Figure 3. *Effect of different thermal treatment times at 42˚C on the cytotoxicity of NK cells. NK cells were heated at 42˚C for 0, 15, 30, 60, 90, 120 and 180 min, and cytotoxicity was evaluated. The error bars represent the standard deviation of the experiments (n=12-24).*

Recovery of NK cell cytotoxicity after thermal treatment. The recovery of NK cell cytotoxicity after thermal injury was examined by heating NK cells at 42˚C for 0, 30, 60, 120 and 180 min; the cells were then incubated for 24, 48 and 72 h at 37˚C. For all thermal treatment times, the major portion of recovery occurred within the first 24 h. An increase in cytotoxicity was significant at all recovery times ($p=0.011$, 0.014 and 0.011, respectively), with a slow increase up to 48 h observed, except for the longest heating time (180 min) (Figure 4A). For a heating time of 30 min, 70% of the cytotoxicity was restored at 72 h.

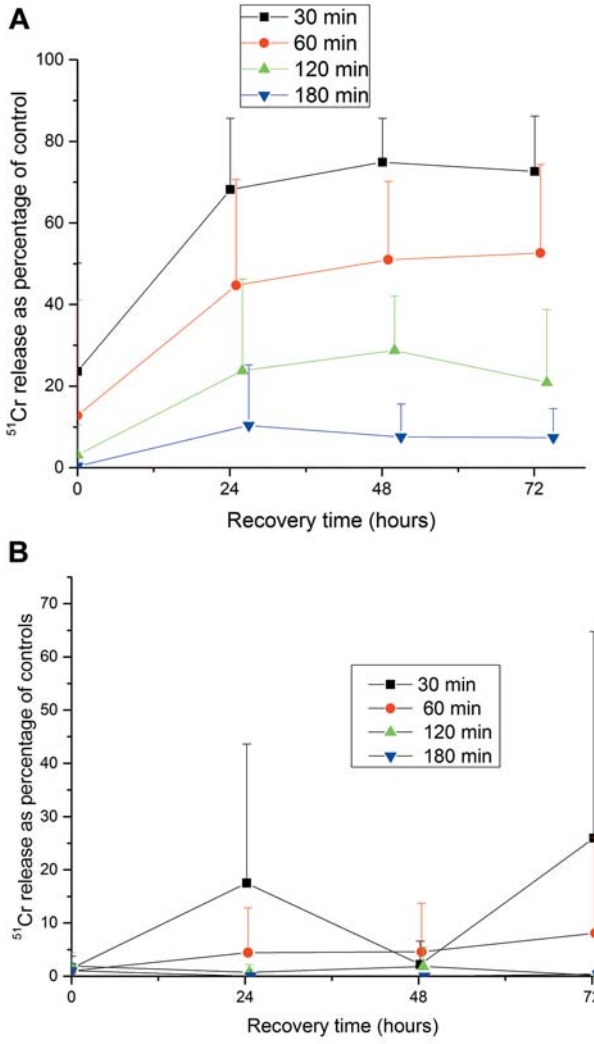

Figure 4. *Recovery of NK cell cytotoxicity after thermal treatment for different times at 42˚C (A) and 45˚C (B). NK cells were incubated at 42˚C (A) and 45˚C (B) for 0, 30, 60, 120 and 180 min. Recovery times were 24, 48 and 72 h at 37˚C. Cytotoxicity was measured. The error bars represent the standard deviation of the experiments (n=4).*

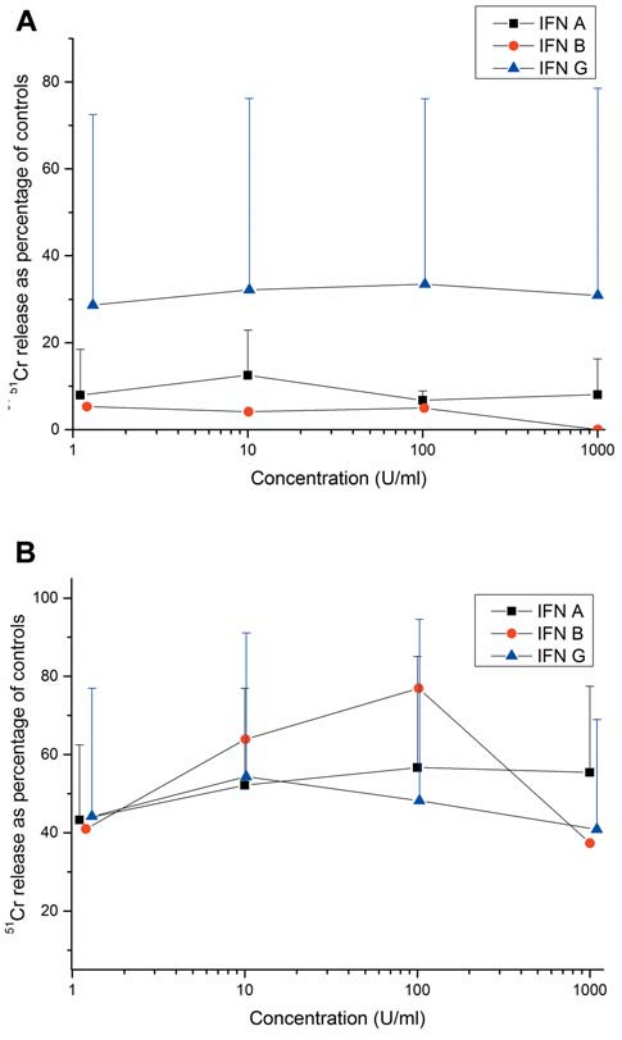

Figure 5. *The role of IFN-α, β, and γ in preventing thermal damage (A) and restoring cytotoxicity (B) to NK cells. The ability of IFN-α,β and γ to prevent thermal damage to NK cells or restore cytotoxicity is presented. Cells were first incubated at 37˚C for 24 hours with IFN α,β and γ at concentrations from 1 to 1000 UI/ml and then treated for 180 min at 42˚C (A). Next, incubation with interferons was conducted after thermal treatment (B). Cytotoxicities were measured. The error bars represent the standard deviation of the experiments (n=2-4).*

The same thermal treatment was conducted at 45˚C, and the restoration of cytotoxicity was much lower than at 42˚C. Indeed, the best recovery of cytotoxicity after 72 h was at most only 25% of the controls after 30 min of thermal treatment (Figure 4B).

The role of IFN α, β, γ and IL-2 in preventing thermal injury and restoring the cytotoxicity of NK cells. To study the role of IFN α, β, and γ in preventing thermal injury, NK cells were incubated for 24 h at 37˚C with 1 to 1000 UI/ml IFN α, β, γ. Thereafter, the cells were heated at 42˚C for 180 min. In these experiments, IFN α, β, and γ exhibited no concentration-dependent effect on cytotoxicity when applied before the thermal treatment (Figure 5A).

Next, the effects of IFN-α, β and γ treatment on NK cell recovery after thermal damage were evaluated, with incubation for 24 h after heating at 42˚C. Again, interferons showed no statistically significant concentration-dependent effects of the recovery of NK cell cytotoxicity (Figure 5B). Although the interferons showed no significant effect on the restoration of NK cell cytotoxicity, recovery was slightly more pronounced when these cytokines were applied after heating.

To explore the ability of IL-2 to prevent thermal damage, the IL-2 concentrations and incubation times necessary for an

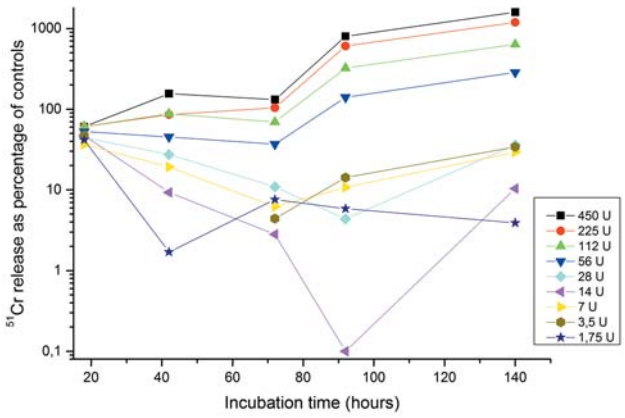

Figure 6. *NK cell cytotoxicity as a function of IL-2 concentration and incubation time at 42˚C before hyperthermia treatment. NK cells were incubated for 0, 18, 42, 72, 92 and 140 hours at 37˚C with IL-2 at concentrations from 0 to 450 U/ml. Subsequently, the cells were subjected to thermal treatment at 4˚C for 3 h. The recovery of cytotoxicity is presented (n=1).*

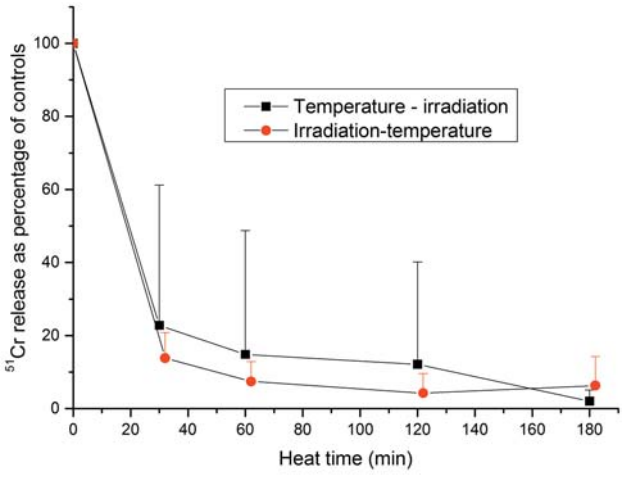

Figure 8. *The effect of the order of different heating times at 42˚C and irradiation on NK cell cytotoxicity. NK cells were treated at 42˚C for different heating times and irradiated with 20 Gy. The following sequences were used: thermal treatment first, then irradiation (temperature-irradiation) and vice versa (irradiation-temperature). The error bars represent the standard deviation of the experiments (n≥6).*

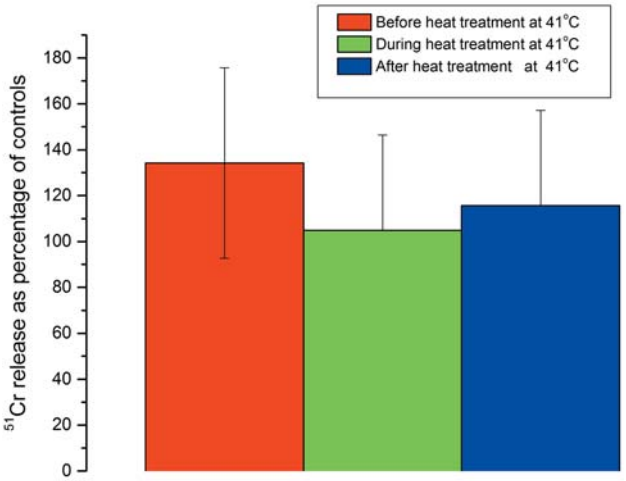

Figure 7. *NK cell cytotoxicity with IL-2 treatment before, during and after thermal treatment at 41˚C. NK cells were incubated with IL-2 at 450 UI/ml for 18 h before, during or after 3 h thermal treatment at a temperature of 41˚C. The error bars represent the standard deviation of the experiments (n=4-5).*

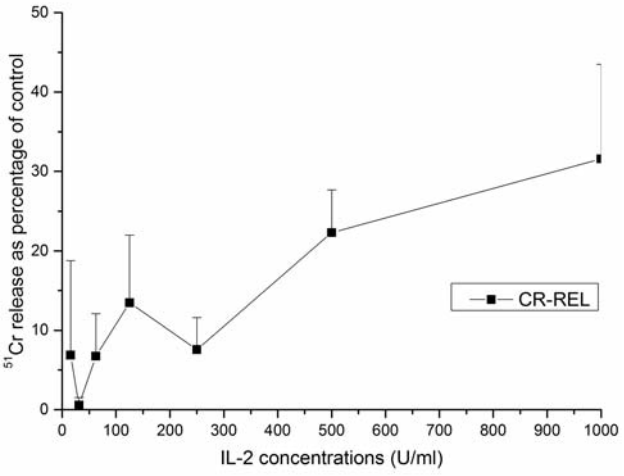

Figure 9. *Effect of different concentrations of IL-2 on NK cell cytotoxicity irradiated with 20 Gy and heated at 42˚C. NK cells were first irradiated with 20 Gy, then heated at 42˚C for 60 min and finally incubated with IL-2 at concentrations from 0 to 1,000 UI/ml. The error bars represent the standard deviation of the experiments (n=3).*

effect were determined. NK cells were incubated from 0 to 140 h with 0 to 450 IU/ml IL-2 and then heated for 180 min at 42˚C. Concentrations of 28 IU/ml or less were not able to fully restore cytotoxicity, whereas concentrations ≥56 IU/ml markedly increased cytotoxicity over 100% of the control. This elevation was strongly dependent on both the IL-2 concentration and incubation time. The highest cytotoxicity value, 1585% of the control, was achieved by using 450 IU/ml IL-2 and an incubation time of 140 h (Figure 6).

Next, we assessed the time of IL-2 treatment in relation to heating by incubating NK cells at 37˚C for 18 h with 450 UI/ml IL-2 before, during or after thermal treatment at 41˚C for 180 min. However, no statistically significant differences between the timing of IL-2 treatment and the heating were observed (p=0.560) (Figure 7).

According to our results, interferons showed a very low and no concentration-dependent effect on NK cell cytotoxicity, whereas IL-2 was clearly effective in a

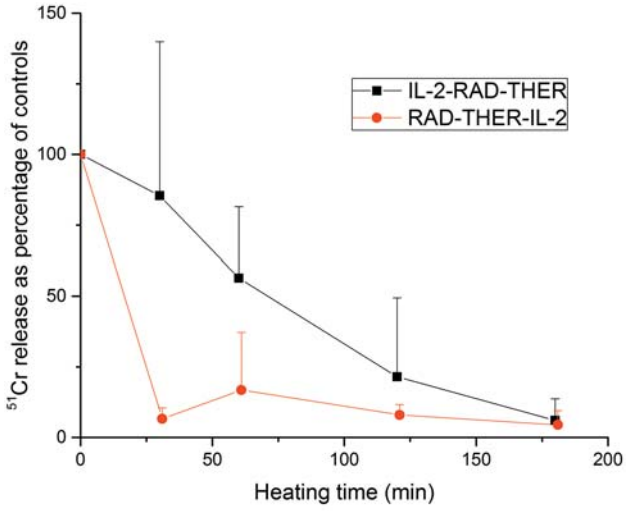

Figure 10. *The cytotoxicity of NK cells treated at 42˚C using combinations of IL-2 treatment, irradiation and hyperthermia. NK cells were incubated with 100 U/I IL-2 for 5 days at 37˚C. The irradiation dose was 20 Gy. Heating times at 42˚C were 0, 30, 60, 120 and 180 min. The following combinations were used: 1) IL-2, irradiation and hyperthermia (IL-2-RAD-THER) and 2) Irradiation, hyperthermia and IL-2 (RAD-THER-IL-2). The error bars represent the standard deviation of the experiments (n=3-12).*

concentration and incubation time-dependent manner. Therefore, IL-2 was the only cytokine selected for the next experiments.

Combined effect of hyperthermia and irradiation on NK cell cytotoxicity. First, NK cells were irradiated with doses from 0 to 30 Gy and then heated at 42˚C for 30 min. Based on its position in the middle of the cytotoxicity dose-response curve, the irradiation dose of 20 Gy was selected for further analysis. NK cells were irradiated at room temperature and then heated at 42˚C for 30, 60, 120 and 180 min. Two approaches were used, either irradiation first-followed by immediate thermal treatment or *vice versa*. No statistically significant differences in NK cell cytotoxicity were observed between the approaches at any heating time (p=0.520) (Figure 8).

In the following experiments, we used the same approaches with 0, 30, 60, 120 and 180 min intervals at 37˚C between the heating and irradiation treatments. Again, we found no statistically significant differences in cytotoxicity at any of the times examined (p=0.280, 0.140, 1.000 and 1.000, respectively).

IL-2 in the recovery of NK cell cytotoxicity from combined hyperthermia and irradiation. The combined effects of irradiation and cytokines on the cytotoxicity of NK cells are described in our earlier study (28). In the present study, we explored whether it was possible to reverse the effects of damage due to combined irradiation and thermal treatment on the cytotoxicity of NK cells.

Firstly, NK cells were incubated for 5 days with 100 UI/ml IL-2, a concentration based on the cytotoxicity dose-response curves of NK cells irradiated, heated and incubated with various IL-2 concentrations (Figure 9). The cells were then irradiated with 20 Gy and heated at 42˚C for 0 to 180 min. The irradiation dose was selected as described above.

Secondly, NK cells were incubated with IL-2 after irradiation and thermal treatment. A significant difference in cytotoxicity was observed when IL-2 was applied before irradiation and heating (p=0.000). Specifically, we found that the restoration of NK cell cytotoxicity was significantly improved with heating times of 30 and 60 min (p=0.030 and 0.000, respectively). However, with longer heating times, the IL-2 in concentration used was not able to significantly prevent the damage caused by irradiation and thermal treatment (Figure 10).

Discussion

We studied the *in vitro* thermal effects on non-selected NK cell cytotoxicity as a function of temperature and with a special focus on heating time and did not observe any significant differences in cytotoxicity within the temperature range from 31 to 37˚C. In our *in vitro* study, we detected neither the immunostimulating effects of acute cold exposure found *in vivo* (25) nor the decrease observed during perioperative hypothermia (26). The most significant decrease in cytotoxicity was observed between 41˚C and 42˚C, and this was strongly dependent on the heating time. In fact, cytotoxicity was almost completely destroyed after heating for 180 min at 42˚C, in agreement with previously published results (18, 31).

After heating times from 0 to 120 min at 42˚C, cytotoxicity was partially recovered, mostly during the first 24 h. However, there was almost no recovery at 45˚C, even with short heating times, as also reported in the literature (20).

In vitro, IFN-α, β or γ showed no dose-dependent effects in preventing or recovering NK cell cytotoxicity from thermal damage, though conflicting results have been published *in vivo*. Robins *et al.* (32) demonstrated that whole-body hyperthermia at 40.5˚C and IFN-γ therapy increases NK cell cytotoxicity, and Payne *et al.* (33) found that mild hyperthermia suppresses interferon-mediated enhancement of NK cell activity in human and murine cells. Conversely, Lamon *et al.* (34) reported the induction of thermotolerance in murine NK cells with IFN-α.

In our study, IL-2 exhibited a dose-dependent enhancement of NK cell cytotoxicity: at concentrations of 56 IU/ml and above, it was capable of fully restoring NK cell cytotoxicity after heating for 180 min at 42˚C. In addition,

there were no significant differences in NK cytotoxicity when IL-2 treatments were applied before, during or after heating. Kappel *et al*. (22) reported similar results at a slightly lower temperature of 39.5˚C.

When treating various cancers using a combination of whole-body heating and irradiation, the temperature usually ranges from 38˚C to 42˚C, and heating times from 40 to 60 min and daily irradiation doses from 1.8-2 Gy (even from 5-10 Gy for superficial tumors) have been applied (13). Thus, the temperature, irradiation and time range of our *in vitro* experiments are the same as those used *in vivo*. In some studies, thermal treatment was applied first, whereas the irradiation treatment was first in others (35). Our approaches included first heating at 42˚C followed by irradiation and vice versa, with no significant differences regarding NK cytotoxicity.

In addition, we investigated whether IL-2 is capable of preventing or restoring NK cell cytotoxicity after damage induced by combined hyperthermia and irradiation, and the recovery from thermal and irradiation damage was significantly higher when IL-2 was applied before heating and irradiation, results that were strongly dependent on heating time. Furthermore, various time intervals between irradiation and heating did not significantly affect the damage to cytotoxicity. We could not find any published study to date, *in vivo* or *in vitro*, on NK cell recovery with IL-2 after combined hyperthermia and irradiation.

The proportion of destroyed normal and tumor cells increases with increasing temperature (7). However, temperatures of 42.0-42.5˚C cannot be exceeded in whole-body cancer treatments. Temperatures up to 42˚C and treatment times from 40 to 60 min per session have been used in whole-body hyperthermia treatment of different cancer types, and an irradiation dose from 1.5 to 2 Gy per session can be added to the treatment. Under these conditions, our *in vitro* results revealed reduced NK cell cytotoxicity that was able to recover at least partially by itself and completely with IL-2. Depending on the individual's age and measurement method, IL-2 levels in the blood of healthy subjects range from 6 to 20 pg/ml (36), values that are less than 1 IU/ml. Theoretically, IL-2 could be applied in patients to reach levels high enough to protect NK cell cytotoxicity from the damage induced by hyperthermia and irradiation. Of course, there are other factors *in vivo* involved in NK cell recovery (8).

Since 1970´s the thermobiological rationale of hyperthermia has developed enormously. Hyperthermia is used clinically to enhance radiation and chemotherapy effects leading to thermoradiobiological and thermoradiochemotherapeutic rationales. Therapeutic whole-body as well as local hyperthermia induces both innate and adaptive anti-tumor immune responses including activation of the NK cells. Substantial roles in increasing the use of hyperthermia and better treatment results are the advanced hyperthermia treatment planning, execution and thermometry (37). In addition, during the last years the importance of immuno-oncology has been re-appraised, when targeted agents could be used to increase antigen dependent cellular cytotoxicity (38) and especially to restore immunological defense against cancer cells (39-42). In addition to IL-2 these new agents might be used during RT to increase cancer cell killing. However, clinical studies are warranted. Rosenberg and co-workers are still investigating better ways to clinically apply personalized immunotherapy for human cancers (43). In their studies the key cell populations are tumor-infiltrating lymphocytes (TILs) and NK cells and one of the key questions is how to increase cytotoxicity of these cells.

Conclusion

The effects of thermal and irradiation treatment on NK cell cytotoxicity were studied *in vitro*. No changes in cytotoxicity were detected at a temperature range from 31˚C to 37˚C, though elevation of the temperature from 41˚C to 42˚C significantly decreased cytotoxicity depending on the heating time. The recovery from the thermal injury by NK cells showed a temperature and heating time-dependent tendency. Although IFN-α, β and γ did not exhibit any concentration-dependent ability to prevent or recover heating damage, IL-2 was able to restore the damage partially or completely in a concentration-dependent manner when applied before, during or after thermal treatment. The impairment of NK cell cytotoxicity by the combination of hyperthermia and irradiation was dependent on the heating temperature and time as well as the irradiation dose, though there was no difference in the loss of cytotoxicity when the hyperthermic treatment was given before irradiation or vice versa. IL-2 was able to restore cytotoxicity more significantly when applied before the combined irradiation and thermal treatment.

Conflicts of Interest

The Authors declare no conflicts of interest.

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